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(54) Title: **LEUCINE-BASED MOTIF AND CLOSTRIDIAL NEUROTOXINS**

(57) Abstract: Modified neurotoxin comprising neurotoxin including structural modification, wherein the structural modification alters the biological persistence, such as the biological half-life and/or a biological activity of the modified neurotoxin relative to an identical neurotoxin without the structural modification. In one embodiment, methods of making the modified neurotoxin include using recombinant techniques. In another embodiment, methods of using the modified neurotoxin to treat conditions include treating various disorders, neuromuscular ailments and pain.

LEUCINE-BASED MOTIF AND CLOSTRIDIAL NEUROTOXINS

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CROSS REFERENCE

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This application is a continuation in part of application serial number 09/620,840, filed July 21, 2000.

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BACKGROUND

The present invention relates to modified neurotoxins, particularly modified Clostridial neurotoxins, and use thereof to treat various conditions including conditions that have been treated using naturally occurring botulinum toxins.

Botulinum toxin, for example, botulinum toxin type A, has been used in the treatment of numerous conditions including pain, skeletal muscle conditions, smooth muscle conditions and glandular conditions. Botulinum toxins are also used for cosmetic purposes.

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Numerous examples exist for treatment using botulinum toxin. For examples of treating pain see Aoki, et al., U.S. Patent 6,113,915 and Aoki, et al., U.S. Patent 5,721,215. For an example of treating a neuromuscular disorder, see U.S. Pat. No. 5,053,005,

which suggests treating curvature of the juvenile spine, i.e., scoliosis, with an acetylcholine release inhibitor, preferably botulinum toxin A. For the treatment of strabismus with botulinum toxin type A, see Elston, J. S., et al., British Journal of Ophthalmology, 1985, 69, 718-724 and 891-896. For the treatment of blepharospasm with botulinum toxin type A, see Adenis, J. P., et al., J. Fr. Ophthalmol., 1990, 13 (5) at pages 259-264. For treating spasmodic and oromandibular dystonia torticollis, see Jankovic et al., Neurology, 1987, 37, 616-623. Spasmodic dysphonia has also been treated with botulinum toxin type A. See Blitzer et al., Ann. Otol. Rhino. Laryngol, 1985, 94, 591-594. Lingual dystonia was treated with botulinum toxin type A according to Brin et al., Adv. Neurol. (1987) 50, 599-608. Cohen et al., Neurology (1987) 37 (Suppl. 1), 123-4, discloses the treatment of writer's cramp with botulinum toxin type A.

It would be beneficial to have botulinum toxins with altered biological persistence and/or altered biological activity. For example, a botulinum toxin can be used to immobilize muscles and prevent limb movements after tendon surgery to facilitate recovery. It would be beneficial to have a botulinum toxin (such as a botulinum toxin type A) which exhibits a reduced period of biological persistence so that a patient can regain muscle use and mobility at about the time they recover from surgery. Furthermore, a botulinum toxin with an altered biological activity, such as an enhanced biological activity can have utility as a more efficient toxin (i.e. more potent per unit amount of toxin), so that less toxin can be used.

Additionally, there is a need for modified neurotoxins (such as modified Clostridial toxins) which can exhibit an enhanced period of biological persistence and modified neurotoxins (such as modified Clostridial toxins) with reduced biological persistence and/or biological activity and methods for preparing such toxins.

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Definitions

Before proceeding to describe the present invention, the following definitions are provided and apply herein.

15

"Heavy chain" means the heavy chain of a Clostridial neurotoxin. It has a molecular weight of about 100 kDa and can be referred to herein as Heavy chain or as H.

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"H_N" means a fragment (having a molecular weight of about 50 kDa) derived from the Heavy chain of a Clostridial neurotoxin which is approximately equivalent to the amino terminal segment of the Heavy chain, or the portion corresponding to that fragment in the intact Heavy chain. It is believed to contain the portion of the natural or wild type Clostridial neurotoxin involved in the translocation of the light chain across an intracellular endosomal membrane.

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"H_C" means a fragment (about 50 kDa) derived from the Heavy chain of a Clostridial neurotoxin which is approximately equivalent to the carboxyl terminal

segment of the Heavy chain, or the portion corresponding to that fragment in the intact Heavy chain. It is believed to be immunogenic and to contain the portion of the natural or wild type Clostridial neurotoxin involved in high affinity binding to various neurons (including motor neurons), and other types of target cells.

"Light chain" means the light chain of a Clostridial neurotoxin. It has a molecular weight of about 50 kDa, and can be referred to as light chain, L or as the proteolytic domain (amino acid sequence) of a Clostridial neurotoxin. The light chain is believed to be effective as an inhibitor of exocytosis, including as an inhibitor of neurotransmitter (i.e. acetylcholine) release when the light chain is present in the cytoplasm of a target cell.

"Neurotoxin" means a molecule that is capable of interfering with the functions of a cell, including a neuron. The "neurotoxin" can be naturally occurring or man-made. The interfered with function can be exocytosis.

"Modified neurotoxin" means a neurotoxin which includes a structural modification. In other words, a "modified neurotoxin" is a neurotoxin which has been modified by a structural modification. The structural modification changes the biological persistence, such as the biological half-life (i.e. the duration of action of the neurotoxin) and/or the

biological activity of the modified neurotoxin relative to the neurotoxin from which the modified neurotoxin is made or derived. The modified neurotoxin is structurally different from a naturally existing neurotoxin.

"Mutation" means a structural modification of a naturally occurring protein or nucleic acid sequence. For example, in the case of nucleic acid mutations, a mutation can be a deletion, addition or substitution of one or more nucleotides in the DNA sequence. In the case of a protein sequence mutation, the mutation can be a deletion, addition or substitution of one or more amino acids in a protein sequence. For example, a specific amino acid comprising a protein sequence can be substituted for another amino acid, for example, an amino acid selected from a group which includes the amino acids alanine, asparagine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine or any other natural or non-naturally occurring amino acid or chemically modified amino acids. Mutations to a protein sequence can be the result of mutations to DNA sequences that when transcribed, and the resulting mRNA translated, produce the mutated protein sequence. Mutations to a protein sequence can also be created by fusing a peptide sequence containing the desired mutation to a desired protein sequence.

"Structural modification" means any change to a neurotoxin that makes it physically or chemically different from an identical neurotoxin without the structural modification.

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"Biological persistence" or "persistence" means the time duration of interference or influence caused by a neurotoxin or a modified neurotoxin with a cellular (such as a neuronal) function, including the
10 temporal duration of an inhibition of exocytosis (such as exocytosis of neurotransmitter, for example, acetylcholine) from a cell, such as a neuron.

"Biological half-life" or "half-life" means the
15 time that the concentration of a neurotoxin or a modified neurotoxin, preferably the active portion of the neurotoxin or modified neurotoxin, for example, the light chain of Clostridial toxins, is reduced to half of the original concentration in a mammalian
20 cell, such as in a mammalian neuron.

"Biological activity" or "activity" means the amount of cellular exocytosis inhibited from a cell per unit of time, such as exocytosis of a
25 neurotransmitter from a neuron.

"Target cell" means a cell (including a neuron) with a binding affinity for a neurotoxin or for a modified neurotoxin.

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SUMMARY

New structurally modified neurotoxins have been discovered. The present structurally modified neurotoxins can provide substantial benefits, for example, enhanced or decreased biological persistence
5 and/or biological half-life and/or enhanced or decreased biological activity as compared to the unmodified neurotoxin.

In accordance with the present invention, there
10 are provided structurally modified neurotoxins, which include a neurotoxin and a structural modification. The structural modification is effective to alter a biological persistence of the structurally modified neurotoxin relative to an identical neurotoxin
15 without the structural modification. Also, the structurally modified neurotoxin is structurally different from a naturally existing neurotoxin.

The present invention also encompasses a modified
20 neurotoxin comprising a neurotoxin with a structural modification, wherein said structural modification is effective to alter a biological activity of said modified neurotoxin relative to an identical neurotoxin without said structural modification, and
25 wherein said modified neurotoxin is structurally different from a naturally existing neurotoxin. This structural modification can be effective to reduce an exocytosis from a target cell by more than the amount of the exocytosis reduced from the target cell by an
30 identical neurotoxin without said structural modification. Alternately, the structural modification can be effective to reduce an exocytosis

from a target cell by less than the amount of the exocytosis reduced from the cell by an identical neurotoxin without said structural modification. Significantly, the exocytosis can be exocytosis of a neurotransmitter and the modified neurotoxin can exhibit an altered biological activity without exhibiting an altered biological persistence. The structural modification can comprise a leucine-based motif. Additionally, the modified neurotoxin can exhibits an altered biological activity as well as an altered biological persistence. The present invention also includes the circumstances where: (a) the modified neurotoxin exhibits an increased biological activity as well as an increased biological persistence; (b) the modified neurotoxin exhibits an increased biological activity and a reduced biological persistence; (c) the modified neurotoxin exhibits a decreased biological activity and a decreased biological persistence, and; (d) the modified neurotoxin exhibits an decreased biological activity and an increased biological persistence.

Importantly, a unit amount (i.e. on a molar basis) of the modified neurotoxin can be more efficient to reduce an exocytosis from a cell than is a unit amount of the naturally existing neurotoxin. In other words, a unit amount of a modified neurotoxin, such as a modified botulinum toxin type A, can cleave its' intracellular substrate (SNAP) in a manner such that a greater inhibition of neurotransmitter exocytosis results (i.e. less

neurotransmitter is released from the cell), as compared to the inhibition of neurotransmitter exocytosis exhibited by the naturally occurring neurotoxin.

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Further in accordance with the present invention, are structurally modified neurotoxins, wherein a structural modification is effective to enhance a biological persistence of the modified neurotoxin. The enhanced biological persistence of the structurally modified neurotoxin can be due, at least in part, to an increased half-life and/or biological activity of the structurally modified neurotoxin.

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Still further in accordance with the present invention, there are provided structurally modified neurotoxins wherein a biological persistence of the structurally modified neurotoxin is reduced relative to that of an identical neurotoxin without the structural modification. This reduction in biological persistence can be due, at least in part, to a decreased biological half-life and/or activity of the structurally modified neurotoxins.

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Still further in accordance with the present invention, there are provided structurally modified neurotoxins wherein the structural modification comprises a number of amino acids. For example, the number of amino acids comprising the structural modification can be 1 or more amino acids, from 1 to

about 22 amino acids, from 2 to about 10 amino acids,
and from about 4 to about 7 amino acids.

In one embodiment, the structural modifications
5 of the structurally modified neurotoxins can comprise
an amino acid. The amino acid can comprise an R
group containing a number of carbons. For example,
the number of carbon atoms in the amino acid can be 1
or more, from 1 to about 20 carbons, from 1 to about
10 12 carbons, from 1 to about 9 carbons, from 2 to
about 6 carbons, and about 4 carbons. R group as
used in this application refers to amino acid side
chains. For example, the R group for alanine is CH_3 ,
and, for example, the R group for serine is CH_2OH .

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In another embodiment, there are provided
structurally modified neurotoxins wherein the
modification comprises an amino acid. The amino
acid can comprise an R group which is substantially
20 hydrocarbyl.

In still another embodiment, there are provided
structurally modified neurotoxins wherein the
structural modification comprises an amino acid. The
25 amino acid further can comprise an R group that
includes at least one heteroatom.

Further in accordance with the present
invention, there are provided structurally modified
30 neurotoxins wherein the structural modification
comprises, for example, a leucine-based motif, a
tyrosin -based motif, and/or an amino acid

derivative. Examples of an amino acid derivative that can comprise a structurally modified neurotoxin are a myristylated amino acid, an N-glycosylated amino acid, and a phosphorylated amino acid. The
5 phosphorylated amino acids can be phosphorylated by, for example, casein kinase II, protein kinase C, and tyrosine kinase.

Still further in accordance with the present
10 invention, there are provided structurally modified neurotoxins which can include a structural modification. The neurotoxin can comprise three amino acid sequence regions. The first region can be effective as a cellular binding moiety. This binding
15 moiety can be a binding moiety for a target cell, such as a neuron. The binding moiety can be the carboxyl terminus of a botulinum toxin heavy chain. It is well known that the carboxyl terminus of a botulinum toxin heavy chain can be effective to bind,
20 for example, receptors found on certain cells, including certain nerve cells. In one embodiment, the carboxyl terminus binds to receptors found on a presynaptic membrane of a nerve cell. The second region can be effective to translocate a structurally
25 modified neurotoxin, or a part of a structurally modified neurotoxin across an endosome membrane. The third region can be effective to inhibit exocytosis from a target cell. The inhibition of exocytosis can be inhibition of neurotransmitter release, such as
30 acetylcholine from a presynaptic membrane. For example, it is well known that the botulinum toxin light chain is effective to inhibit, for example,

acetylcholin (as well as other neurotransmitters) release from various neuronal and non-neuronal cells.

At least one of the first, second or third
5 regions can be substantially derived from a Clostridial neurotoxin. The third region can include the structural modification. In addition, the modified neurotoxin can be structurally different from a naturally existing neurotoxin. Also, the
10 structural modification can be effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification.

15 In one embodiment, there are provided structurally modified neurotoxins, wherein the neurotoxin can be botulinum serotype A, B, C₁, C₂, D, E, F, G, tetanus toxin and/or mixtures thereof.

20 In another embodiment, there are provided structurally modified neurotoxins where the third region can be derived from botulinum toxin serotype A. In addition, there are provided structurally modified neurotoxins wherein the third region can not
25 be derived from botulinum serotype A.

In still another embodiment, there are provided structurally modified neurotoxins wherein the structural modification includes a biological
30 persistence enhancing component effective to enhance the biological persistence of the structurally modified neurotoxin. The enhancing of the biological

persistence can be at least in part due to an increase in biological half-life and/or activity of the structurally modified neurotoxin.

5 Further in accordance with the present invention, there are provided structurally modified neurotoxins comprising a biological persistence enhancing component, wherein the biological persistence enhancing component can comprise a
10 leucine-based motif. The leucine-based motif can comprise a run of 7 amino acids, where a quintet of amino acids and a duplet of amino acids can comprise the leucine-based motif. The quintet of amino acids can define the amino terminal end of the leucine-
15 based motif. The duplet of amino acids can define the carboxyl end of the leucine-based motif. There are provided structurally modified neurotoxins wherein the quintet of amino acids can comprise one or more acidic amino acids. For example, the acidic
20 amino acid can be glutamate or aspartate. The quintet of amino acids can comprise a hydroxyl containing amino acid. The hydroxyl containing amino acid can be, for example, a serine, a threonine or a tyrosine. This hydroxyl containing amino acid can be
25 phosphorylated. At least one amino acid comprising the duplet of amino acids can be a leucine, isoleucine, methionine, alanine, phenylalanine, tryptophan, valine or tyrosine. In addition, the duplet of amino acids in the leucine-based motif can
30 be leucine-leucine, leucine-isoleucine, isoleucine-leucine or isoleucine-isoleucine, leucine-methionine. The leucine-based motif can be an amino acid sequence

of phenylalanine-glutamate-phenylalanine-tyrosine-lysine-leucine-leucine.

In one embodiment, there are provided
5 structurally modified neurotoxins wherein the modification can be a tyrosine-based motif. The tyrosine-based motif can comprise four amino acids. The amino acid at the N-terminal end of the tyrosine-based motif can be a tyrosine. The amino acid at the
10 C-terminal end of the tyrosine-based motif can be a hydrophobic amino acid.

Further in accordance with the present invention, the third region can be derived from
15 botulinum toxin serotype A or from one of the other botulinum toxin serotypes.

Still further in accordance with the present invention, there are provided structurally modified
20 neurotoxins where the biological persistence of the structurally modified neurotoxin can be reduced relative to an identical neurotoxin without the structural modification. The reduced biological persistence can be in part due a decreased biological
25 half-life and/or to a decrease biological activity of the neurotoxin.

In one embodiment, there are provided structurally modified neurotoxins, where the
30 structural modification can include a leucine-based motif with a mutation of one or more amino acids comprising the leucine-based motif. The mutation can

be a deletion or substitution of one or more amino acids of the leucine-based motif.

In another embodiment, there are provided
5 structurally modified neurotoxins, where the structural modification includes a tyrosine-based motif with a mutation of one or more amino acids comprising the tyrosine-based motif. For example, the mutation can be a deletion or substitution of one
10 or more amino acids of the tyrosine-based motif.

In still another embodiment, there are provided structurally modified neurotoxins, wherein the structural modification comprises an amino acid
15 ~~derivative with a mutation of the amino acid derivative or a mutation to a nucleotide or amino acid sequence which codes for the derivativization of the amino acid.~~ For example, a deletion or substitution of the derivatized amino acid or a
20 nucleotide or amino acid sequence responsible for a derivatization of the derivatized amino acid. The amino acid derivative can be, for example, a myristylated amino acid, an N-glycosylated amino acid, or a phosphorylated amino acid. The
25 phosphorylated amino acid can be produced by, for example, casein kinase II, protein kinase C or tyrosine kinase.

In one embodiment of the present invention,
30 there are provided structurally modified neurotoxins, wherein the first, second and/or third regions of the

structurally modified neurotoxins can be produced by recombinant DNA methodologies, i.e. produced recombinantly.

5 In another embodiment of the present invention, there are provided structurally modified neurotoxins, wherein the first, second and/or third region of the neurotoxin is isolated from a naturally existing Clostridial neurotoxin.

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Another embodiment of the present invention provides a modified neurotoxin comprising a botulinum toxin (such as a botulinum toxin type A) which includes a structural modification which is effective
15 to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification can comprise a deletion of amino acids 416 to 437 from a light chain of the neurotoxin (Fig.
20 3).

In still another embodiment of the present invention there is provided a modified neurotoxin (such as a botulinum toxin type A) which includes a
25 structural modification which is effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification can comprise a deletion of amino acids 1 to 8 from a
30 light chain of the neurotoxin (Fig. 3).

Still further in accordance with the present invention there is provided a modified neurotoxin, such as a botulinum toxin type A, which includes a structural modification which is effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without the structural modification. The structural modification can comprise a deletion of amino acids 1 to 8 and 416 to 437 from a light chain of the neurotoxin (Fig. 3).

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Still further in accordance with the present invention, there is provided a modified botulinum toxin, such as a modified botulinum toxin type A, which includes a structural modification effective to alter a biological persistence of the modified neurotoxin relative to an identical neurotoxin without said structural modification. The structural modification can comprise a substitution of leucine at position 427 for an alanine and a substitution of leucine at position 428 for an alanine in a light chain of said neurotoxin (Fig. 3).

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Additionally, the scope of the present invention also includes methods for enhancing the biological persistence and/or or for enhancing the biological activity of a neurotoxin. In these methods, a structural modification can be fused or added to the neurotoxin, for example, the structural modification can be a biological persistence enhancing component and/or a biological activity enhancing component. Examples of structural modifications that can be fused or added to the neurotoxin are a leucine-based

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motif, a tyrosine-based motif and an amino acid derivative. Examples of amino acid derivatives are a myristylated amino acid, an N-glycosylated amino acid, and a phosphorylated amino acid. An amino acid
5 can be phosphorylated by, for example, protein kinase C, casein kinase II or tyrosine kinase.

Also in accordance with the present invention, there are provided methods for reducing the
10 biological persistence and/or for reducing the biological activity of a neurotoxin. These methods can comprise a step of mutating an amino acid of the neurotoxin. For example, an amino acid of a leucine-based motif within the neurotoxin can be mutated.
15 Also, for example, one or more amino acids within a tyrosine-based motif of the neurotoxin can be mutated. Also, for example, an amino acid derivative for DNA or amino acid sequence responsible for the derivatization of the amino acid can be mutated. The
20 derivatized amino acid can be a myristylated amino acid, a N-glycosylated amino acid, or a phosphorylated amino acid. The phosphorylated amino acid can be produced by, for example, protein kinase C, casein kinase II and tyrosine kinase. These
25 mutations can be, for example, amino acid deletions or amino acid substitutions.

The present invention also includes methods for treating a condition. The methods can comprise a
30 step of administering an effective dose of a structurally modified neurotoxin to a mammal to treat a condition. The structurally modified neurotoxin

can include a structural modification. The structural modification is effective to alter the biological persistence and/or the biological activity of the neurotoxin. These methods for treating a condition

5 can utilize a neurotoxin that does not comprise a leucine-based motif. Also, these methods for treating a condition can utilize a neurotoxin, which includes a biological persistence enhancing component and/or a biological activity enhancing component .

10 The biological persistence or activity enhancing component can comprise, for example, a tyrosine-based motif, a leucine-based motif or an amino acid derivative. The amino acid derivative can be, for example, a myristylated amino acid, an N-glycosylated

15 ~~amino acid or a phosphorylated amino acid.~~ The phosphorylated amino acid can be produced by, for example, protein kinase C, casein kinase II or tyrosine kinase. The condition treated can be a neuromuscular disorder, an autonomic disorder or

20 pain. The treatment of a neuromuscular disorder can comprise a step of locally administering an effective amount of a modified neurotoxin to a muscle or a group of muscles. A method for treating an autonomic disorder can comprise a step of locally administering

25 an effective amount of a modified neurotoxin to a gland or glands. A method for treating pain can comprise a step of administering an effective amount of a modified neurotoxin to the site of the pain. In addition, the treatment of pain can comprise a step

30 of administering an effective amount of a modified neurotoxin to the spinal cord.

Still further in accordance with the present invention, there are provided methods for treating with modified neurotoxins conditions including spasmodic dysphonia, laryngeal dystonia, 5 oromandibular dysphonia, lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, 10 tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhydrosis, excessive salivation, excessive gastrointestinal secretions, pain from muscle spasms, headache pain, brow furrows and skin wrinkles.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows localization of GFP-botulinum toxin A light chain in (nerve growth factor) NGF- 20 differentiated live PC12 cells visualized on a fluorescence inverted microscope.

Fig. 2 shows the localization of GFP-truncated botulinum toxin A light chain in NGF-differentiated 25 live PC12 cells visualized on a fluorescence inverted microscope.

Fig. 3 shows the amino acid sequence for botulinum type A light chain. The amino acid sequence 30 shown, minus the underlined amino acids represents botulinum type A truncated light chain.

Fig. 4 shows the localization of GFP-botulinum toxin A light chain with LL to AA mutation at position 427 and 428 in NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope.

Fig. 5 shows localization of fluorescently labeled anti-SNAP-25 visualized in horizontal confocal sections of staurosporine-differentiated PC12 cells.

Fig. 6 shows an X-ray crystallographic structure of botulinum toxin type A.

Fig. 7 shows localization of GFP-botulinum type B neurotoxin light chain in NGF-differentiated live PC12 cells visualized on a fluorescence inverted microscope.

Fig. 8 shows sequence alignment and consensus sequence for botulinum toxin type A HallA light chain and botulinum toxin type B Danish I light chain.

Fig. 9 is a graph which illustrates the results of an in vitro ELISA assay carried out by the inventors demonstrating that a truncated LC/A in vitro cleaves substrate at a slower rate or less efficiently than does non-truncated LC/A.

Fig 10 shows a comparison of LC/A constructs expressed from E. coli for in vitro analysis.

DETAILED DESCRIPTION

The present invention is based upon the
5 discovery that the biological persistence and/or the
biological activity of a neurotoxin can be altered by
structurally modifying the neurotoxin. In other
words, a modified neurotoxin with an altered
biological persistence and/or biological activity can
10 be formed from a neurotoxin containing or including a
structural modification. In one embodiment, the
structural modification includes the fusing of a
biological persistence enhancing component to the
primary structure of a neurotoxin to enhance its
15 biological persistence. In a preferred embodiment,
the biological persistence enhancing component is a
leucine-based motif. Even more preferably, the
biological half-life and/ or the biological activity
of the modified neurotoxin is enhanced by about 100%.
20 Generally speaking, the modified neurotoxin has a
biological persistence of about 20% to 300% more than
an identical neurotoxin without the structural
modification. That is, for example, the modified
neurotoxin including the biological persistence
25 enhancing component is able to cause a substantial
inhibition of neurotransmitter release for example,
acetylcholine from a nerve terminal for about 20% to
about 300% longer than a neurotoxin that is not
modified.

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The present invention also includes within its
scope a modified neurotoxin with a biological

activity altered as compared to the biological activity of the native or unmodified neurotoxin. For example, the modified neurotoxin can exhibit a reduced or an enhanced inhibition of exocytosis (such as exocytosis of a neurotransmitter) from a target cell with or without any alteration in the biological persistence of the modified neurotoxin.

In a broad embodiment of the present invention, a leucine-based motif is a run of seven amino acids. The run is organized into two groups. The first five amino acids starting from the amino terminal of the leucine-based motif form a "quintet of amino acids." The two amino acids immediately following the quintet of amino acids form a "duplet of amino acids." In a preferred embodiment, the duplet of amino acids is located at the carboxyl terminal region of the leucine-based motif. In another preferred embodiment, the quintet of amino acids includes at least one acidic amino acid selected from a group consisting of a glutamate and an aspartate.

The duplet of amino acid includes at least one hydrophobic amino acid, for example leucine, isoleucine, methionine, alanine, phenylalanine, tryptophan, valine or tyrosine. Preferably, the duplet of amino acid is a leucine-leucine, a leucine-isoleucine, an isoleucine-leucine or an isoleucine-isoleucine, leucine-methionine. Even more preferably, the duplet is a leucine-leucine.

In one embodiment, the leucine-based motif is xDxxxLL, wherein x can be any amino acids. In another embodiment, the leucine-based motif is xExxxLL, wherein E is glutamic acid. In another
5 embodiment, the duplet of amino acids can include an isoleucine or a methionine, forming xDxxxLI or xDxxxLM, respectively. Additionally, the aspartic acid, D, can be replaced by a glutamic acid, E, to form xExxxLI, xExxxIL and xExxxLM. In a preferred
10 embodiment, the leucine-based motif is phenylalanine-glutamate-phenylalanine-tyrosine-lysine-leucine-leucine, SEQID #1.

In another embodiment, the quintet of amino
15 acids comprises at least one hydroxyl containing amino acid, for example, a serine, a threonine or a tyrosine. Preferably, the hydroxyl containing amino acid can be phosphorylated. More preferably, the hydroxyl containing amino acid is a serine which can
20 be phosphorylated to allow for the binding of adapter proteins.

Although non-modified amino acids are provided as examples, a modified amino acid is also
25 contemplated to be within the scope of this invention. For example, leucine-based motif can include a halogenated, preferably, fluorinated leucine.

30 Various leucine-based motifs are found in various species. A list of possible leucine-based motifs derived from the various species that can be used in

accordance with this invention is shown in Table 1.
This list is not intended to be limiting.

TABLE 1

	<u>Species</u>	<u>Sequence</u>	<u>SEQID#</u>
5	Botulinum type A	FEFYKLL	1
	Rat VMAT1	EEKRAIL	2
	Rat VMAT 2	EEKMAIL	3
10	Rat VACht	SERDVLL	4
	Rat δ	VDTQVLL	5
	Mouse δ	AEVQALL	6
	Frog γ/δ	SDKQNLL	7
	Chicken γ/δ	SDRQNLI	8
15	Sheep δ	ADTQVLM	9
	Human CD3 γ	SDKQTLL	10
	Human CD4	SQIKRLL	11
	Human δ	ADTQALL	12
20	<i>S. cerevisiae</i> Vam3p	NEQSPLL	13

VMAT is vesicular monoamine transporter; VACht is vesicular acetylcholine transporter and *S. cerevisiae* Vam3p is a yeast homologue of synaptobrevin.
25 Italicized serine residues are potential sites of phosphorylation.

The modified neurotoxin can be formed from any neurotoxin. Also, the modified neurotoxin can be
30 formed from a fragment of a neurotoxin, for example, a botulinum toxin with a portion of the light chain

and/or heavy chain removed. Preferably, the neurotoxin used is a Clostridial neurotoxin. A Clostridial neurotoxin comprises a polypeptide having three amino acid sequence regions. The first amino acid sequence region can include a target cell (i.e. a neuron) binding moiety which is substantially completely derived from a neurotoxin selected from a group consisting of beratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C₁, D, E, F, and G. Preferably, the first amino acid sequence region is derived from the carboxyl terminal region of a toxin heavy chain, H_C. Also, the first amino acid sequence region can comprise a targeting moiety which can comprise a molecule (such as an amino acid sequence) that can bind to a receptor, such as a cell surface protein or other biological component on a target cell.

The second amino acid sequence region is effective to translocate the polypeptide or a part thereof across an endosome membrane into the cytoplasm of a neuron. In one embodiment, the second amino acid sequence region of the polypeptide comprises an amine terminal of a heavy chain, H_N, derived from a neurotoxin selected from a group consisting of beratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C₁, D, E, F, and G.

The third amino acid sequence region has therapeutic activity when it is released into the cytoplasm of a target cell, such as a neuron. In one embodiment, the third amino acid sequence region of

the polypeptide comprises a toxin light chain, L, derived from a neurotoxin selected from a group consisting of beratti toxin; butyricum toxin; tetanus toxin; botulinum type A, B, C₁, D, E, F, and G.

5

The Clostridial neurotoxin can be a hybrid neurotoxin. For example, each of the neurotoxin's amino acid sequence regions can be derived from a different Clostridial neurotoxin serotype. For example, in one embodiment, the polypeptide comprises a first amino acid sequence region derived from the H_C of the tetanus toxin, a second amino acid sequence region derived from the H_N of botulinum type B, and a third amino acid sequence region derived from the light chain of botulinum serotype E. All other possible combinations are included within the scope of the present invention.

Alternatively, all three of the amino acid sequence regions of the Clostridial neurotoxin can be from the same species and same serotype. If all three amino acid sequence regions of the neurotoxin are from the same Clostridial neurotoxin species and serotype, the neurotoxin will be referred to by the species and serotype name. For example, a neurotoxin polypeptide can have its first, second and third amino acid sequence regions derived from Botulinum type E. In which case, the neurotoxin is referred as Botulinum type E.

30

Additionally, each of the three amino acid sequence regions can be modified from the naturally occurring sequence from which they are derived. For example, the amino acid sequence region can have at least one or more amino acids added or deleted as compared to the naturally occurring sequence.

A biological persistence enhancing component or a biological activity enhancing component, for example a leucine-based motif, can be fused with any of the above described neurotoxins to form a modified neurotoxin with an enhanced biological persistence and/or an enhanced biological activity. "Fusing" as used in the context of this invention includes covalently adding to or covalently inserting in between a primary structure of a neurotoxin. For example, a biological persistence enhancing component and/or a biological activity enhancing component can be added to a Clostridial neurotoxin which does not have a leucine-based motif in its primary structure. In one embodiment, a leucine-based motif is fused with a hybrid neurotoxin, wherein the third amino acid sequence is derived from botulinum serotype A, B, C₁, C₂, D, E, F, or G. In another embodiment, the leucine-based motif is fused with a botulinum type E.

In another embodiment, a biological persistence enhancing component and/or a biological activity enhancing component is added to a neurotoxin by altering a cloned DNA sequence encoding the neurotoxin. For example, a DNA sequence encoding a biological persistence enhancing component and/or a

biological activity enhancing component is added to a cloned DNA sequence encoding the neurotoxin into which the biological persistence enhancing component and/or a biological activity enhancing component is to be added. This can be done in a number of ways which are familiar to a molecular biologist of ordinary skill. For example, site directed mutagenesis or PCR cloning can be used to produce the desired change to the neurotoxin encoding DNA sequence. The DNA sequence can then be reintroduced into a native host strain. In the case of botulinum toxins the native host strain would be a Clostridium botulinum strain. Preferably, this host strain will be lacking the native botulinum toxin gene. In an alternative method, the altered DNA can be introduced into a heterologous host system such as E. coli or other prokaryotes, yeast, insect cell lines or mammalian cell lines. Once the altered DNA has been introduced into its host, the recombinant toxin containing the added biological persistence enhancing component and/or a biological activity enhancing component can be produced by, for example, standard fermentation methodologies.

Similarly, a biological persistence enhancing component can be removed from a neurotoxin. For example, site directed mutagenesis can be used to eliminate biological persistence enhancing components, for example, a leucine-based motif.

Standard molecular biology techniques that can be used to accomplish these and other genetic

manipulations are found in Sambrook et al. (1989) which is incorporated in its entirety herein by reference.

5 In one embodiment, the leucine-based motif is fused with, or added to, the third amino acid sequence region of the neurotoxin. In a preferred embodiment, the leucine-based motif is fused with, or added to, the region towards the carboxylic terminal
10 of the third amino acid sequence region. More preferably, the leucine-based motif is fused with, or added to, the carboxylic terminal of the third region of a neurotoxin. Even more preferably, the leucine-based motif is fused with, or added to the carboxylic
15 terminal of the third region of botulinum type E. The third amino acid sequence to which the leucine-based motif is fused or added can be a component of a hybrid or chimeric modified neurotoxin. For example, the leucine-based motif can be fused to or added to
20 the third amino acid sequence region (or a part thereof) of one botulinum toxin type (i.e. a botulinum toxin type A), where the leucine-based motif-third amino acid sequence region has itself been fused to or conjugated to first and second amino
25 acid sequence regions from another type (or types) of a botulinum toxin (such as botulinum toxin type B and/or E).

30 In another embodiment, a structural modification of a neurotoxin which has a pre-existing biological persistence enhancing component and/or a biological activity enhancing component, for example, a leucine-

based motif includes deleting or substituting one or more amino acids of the leucine-based motif. In addition, a modified neurotoxin includes a structural modification which results in a neurotoxin with one
5 or more amino acids deleted or substituted in the leucine-based motif. The removal or substitution of one or more amino acids from the preexisting leucine-based motif is effective to reduce the biological persistence and/or a biological activity of a
10 modified neurotoxin. For example, the deletion or substitution of one or more amino acids of the leucine-based motif of botulinum type A reduces the biological half-life and/or the biological activity of the modified neurotoxin.

15

Amino acids that can be substituted for amino acids contained in a biological persistence enhancing component include alanine, asparagine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine,
20 histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, arginine, serine, threonine, valine, tryptophan, tyrosine and other naturally occurring amino acids as well as non-standard amino acids.

25

In the present invention the native botulinum type A light chain has been shown to localize to differentiated PC12 cell membranes in a characteristic pattern. Biological persistence
30 enhancing components are shown to substantially contribute to this localization.

The data of the present invention demonstrates that when the botulinum toxin type A light chain is truncated or when the leucine-based motif is mutated, the light chain substantially loses its ability to
5 localize to the membrane in its characteristic pattern. Localization to the cellular membrane is believed to be a key factor in determining the biological persistence and/or the biological activity of a botulinum toxin. This is because localization
10 to a cell membrane can protect the localized protein from inter-cellular protein degrading.

Figures 1 and 2 show that deletion of the leucine-based motif from the light chain of botulinum
15 type A can change membrane localization of the type A light chain. Figure 1 shows localization of GFP-light chain A fusion protein in differentiated PC12 cells. The GFP fusion proteins were produced and visualized in differentiated PC12 cells using methods
20 well known to those skilled in the art, for example, as described in Galli et al (1998) Mol Biol Cell 9:1437-1448, incorporated in its entirety herein by reference; also, for example, as described in Martinez-Arca et al (2000) J Cell Biol 149:889-899,
25 also incorporated in its entirety herein by reference. Localization of a GFP-truncated light chain A is shown in figure 2. Comparing figures 1 and 2, it can be seen that the pattern of localization is completely altered by the deletion of
30 the N-terminus and C-terminus comprising the leucine-based motif. Figure 3 shows the amino acid sequence of the botulinum type A light chain. The underlined

amino acid sequences indicate the amino acids that were deleted in the truncated mutant. The leucin - based motif is indicated by the asterisked bracket.

5 Further studies have been done in the present invention to analyze the effect of specific amino acid substitutions within the leucine-based motif. For example, in one study both leucine residues contained in the leucine-based motif were substituted
10 for alanine residues. Figure 4 shows the fluorescent image of differentiated PC12 cells transfected with DNA encoding this di-leucine to di-alanine substituted GFP-botulinum A light chain. As can be seen, the substitution of alanine for leucine, at
15 ~~positions 427 and 428 in the botulinum type A light chain~~ substantially changes the localization characteristic of the light chain.

It is within the scope of this invention that a
20 leucine-based motif, or any other persistence enhancing component and/or a biological activity enhancing component present on a light chain, can be used to protect the heavy chain as well. A random coil belt extends from the botulinum type A
25 translocation domain and encircles the light chain. It is possible that this belt keeps the two subunits in proximity to each other inside the cell while the light chain is localized to the cell membrane. The structure of native botulinum toxin type A is shown
30 in figure 6.

In addition, the data of the present invention shows that the leucine-based motif can be valuable in localizing the botulinum A toxin in close proximity to the SNAP-25 substrate within the cell. This can
5 mean that the leucine-based motif is important not only for determining the half-life of the toxin but for determining the activity of the toxin as well. That is, the toxin will have a greater activity if it is maintained in close proximity to the SNAP-25
10 substrate inside the cell. Figure 5 shows the localization of SNAP-25 in horizontal confocal sections of differentiated PC12 cells (from Martinez-Arca et al (2000) J Cell Biol 149:889-899). Similarity in the pattern of localization can be seen
15 when comparing localization of botulinum type A light chain as seen in figure 1 to localization of SNAP-25 seen in figure 5.

The data of the present invention clearly shows
20 that truncation of the light chain, thereby deleting the leucine-based motif, or amino acid substitution within the leucine-based motif substantially changes membrane localization of the botulinum type A light chain in nerve cells. In both truncation and
25 substitution a percentage of the altered light chain can localize to the cell membrane in a pattern unlike that of the native type A light chain (see figures 1, 2 and 4). This data supports the presence of biological persistence enhancing components other
30 than a leucine-based motif such as tyrosine motifs and amino acid derivatives. Use of these other biological persistence enhancing components and/or a

biological activity enhancing components in modified neurotoxins is also within the scope of the present invention.

5 Also within the scope of the present invention is more than one biological persistence enhancing component used in combination in a modified neurotoxin to alter biological persistence of the neurotoxin that is modified. The present invention
10 also includes use of more than one biological activity enhancing or biological activity reducing components used in combination in a modified neurotoxin to alter the biological activity of the neurotoxin that is modified.

15

 Tyrosine-based motifs are within the scope of the present invention as biological persistence and/or a biological activity altering components. Tyrosine-based motifs comprise the sequence Y-X-X-Hy
20 where Y is tyrosine, X is any amino acid and Hy is a hydrophobic amino acid. Tyrosine-based motifs can act in a manner that is similar to that of leucine-based motifs. In figure 3 some of tyrosine motifs found in the type A toxin light chain are bracketed.
25 In addition, a tyrosine-based motif is found within the leucine-based motif which is indicated by an asterisked bracket in figure 3.

 Also within the scope of the present invention
30 are modified neurotoxins which comprise one or more biological persistence altering components and/or a

biological activity enhancing components which occur naturally in both botulinum toxin types A and B.

Figure 7 shows localization of GFP-botulinum
5 type B neurotoxin light chain in live, differentiated
PC12 cells. Localization of the type B light chain
appears to be to an intracellular organelle. Similar
localization pattern is seen for GFP-truncated
botulinum type A shown in figure 2. Localization of
10 a botulinum toxin, or botulinum toxin light chain,
within the cell is believed to be a key factor in
determining biological persistence and/or biological
activity of the toxin. Therefore, these data appear
to indicate that there are biological persistence
15 altering component(s), and/or biological activity
altering component(s), common to the type A and type
B botulinum toxins. These, and other biological
persistence altering components, and biological
activity altering components, are contemplated for
20 use in accordance with the present invention.

Figure 8 shows a sequence alignment between type
A and type B light chains isolated from strains type
A HallA (SEQ ID NO: 19) and type B Danish I (SEQ ID
25 NO: 20) respectively. Light chains or heavy chains
isolated from other strains of botulinum toxin types
A and B can also be used for sequence comparison.
The shaded amino acids represent amino acid
identities, or matches, between the chains. Each of
30 the shaded amino acids between amino acid position 10
and amino acid position 425 of the figure 8 consensus
sequence, alone or in combination with any other

shaded amino acid or amino acids, represents a biological persistence altering component that is within the scope of the present invention. For example, amino acids KAFK at positions 19 to 22, LNK
5 at positions 304 to 306, L at position 228 in combination with KL at positions 95 and 96, FDKLYK at positions 346 to 351, YL-T at positions 78 to 81, YYD at positions 73 to 75 in combination with YL at positions 78 and 79 in combination with T a position
10 81, F at position 297 in combination with I at position 300 in combination with KL at positions 95 and 96 can be biological persistence altering components for use within the scope of this invention. In addition, conserved regions of charge,
15 hydrophobicity, hydro-philicity and/or conserved secondary, tertiary, or quaternary structures that may be independent of conserved sequence are within the scope of the present invention.

20 Amino acid derivatives are also within the scope of the present invention as biological persistence enhancing components and/or as biological activity enhancing components. Examples of amino acid derivatives that act to effect biological persistence
25 and/or biological activity are phosphorylated amino acids. These amino acids include, for example, amino acids phosphorylated by tyrosine kinase, protein kinase C or casein kinase II. Other amino acid derivatives within the scope of the present invention
30 as biological persistence enhancing components and/or as biological activity enhancing components are

myristylated amino acids and N-glycosylated amino acids.

In one broad aspect of the present invention, a method is provided for treating a condition using a modified neurotoxin. The conditions can include, for example, skeletal muscle conditions, smooth muscle conditions, pain and glandular conditions. The modified neurotoxin can also be used for cosmetics, for example, to treat brow furrows.

The neuromuscular disorders and conditions that can be treated with a modified neurotoxin include: for example, spasmodic dysphonia, laryngeal dystonia, oromandibular and lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorders, spasmodic torticollis, cerebral palsy, focal spasticity and other voice disorders, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia and other muscle tone disorders and other disorders characterized by involuntary movements of muscle groups can be treated using the present methods of administration. Other examples of conditions that can be treated using the present methods and compositions are lacrimation, hyperhidrosis, excessive salivation and excessive gastrointestinal secretions, as well as other secretory disorders. In addition, the present invention can be used to treat dermatological conditions, for example, reduction of brow furrows,

reduction of skin wrinkles. The present invention can also be used in the treatment of sports injuries.

5 Borodic U.S. Patent No. 5,053,005 discloses methods for treating juvenile spinal curvature, i.e. scoliosis, using botulinum type A. The disclosure of Borodic is incorporated in its entirety herein by reference. In one embodiment, using substantially
10 similar methods as disclosed by Borodic, a modified neurotoxin can be administered to a mammal, preferably a human, to treat spinal curvature. In a preferred embodiment, a modified neurotoxin comprising botulinum type E fused with a leucine-
15 based motif is administered. Even more preferably, a modified neurotoxin comprising botulinum type A-E with a leucine-based motif fused to the carboxyl terminal of its light chain is administered to the mammal, preferably a human, to treat spinal
20 curvature.

In addition, the modified neurotoxin can be administered to treat other neuromuscular disorders using well known techniques that are commonly
25 performed with botulinum type A. For example, the present invention can be used to treat pain, for example, headache pain, pain from muscle spasms and various forms of inflammatory pain. For example, Aoki U.S. Patent NO: 5,721,215 and Aoki U.S. Patent
30 No: 6,113,915 disclose methods of using botulinum toxin type A for treating pain. The disclosure of

these two patents is incorporated in its entirety herein by reference.

Autonomic nervous system disorders can also be
5 treated with a modified neurotoxin. For example,
glandular malfunctioning is an autonomic nervous
system disorder. Glandular malfunctioning includes
excessive sweating and excessive salivation.
Respiratory malfunctioning is another example of an
10 autonomic nervous system disorder. Respiratory
malfunctioning includes chronic obstructive pulmonary
disease and asthma. Sanders et al. disclose methods
for treating the autonomic nervous system; for
example, treating autonomic nervous system disorders
15 such as excessive sweating, excessive salivation,
asthma, etc., using naturally existing botulinum
toxins. The disclosure of Sander et al. is
incorporated in its entirety by reference herein. In
one embodiment, substantially similar methods to that
20 of Sanders et al. can be employed, but using a
modified neurotoxin, to treat autonomic nervous
system disorders such as the ones discussed above.
For example, a modified neurotoxin can be locally
applied to the nasal cavity of the mammal in an
25 amount sufficient to degenerate cholinergic neurons
of the autonomic nervous system that control the
mucous secretion in the nasal cavity.

Pain that can be treated by a modified
30 neurotoxin includes pain caused by muscle tension, or
spasm, or pain that is not associated with muscle
spasm. For example, Binder in U.S. Patent No.

5,714,468 discloses that headache caused by vascular disturbances, muscular tension, neuralgia and neuropathy can be treated with a naturally occurring botulinum toxin, for example Botulinum type A. The disclosures of Binder are incorporated in its entirety herein by reference. In one embodiment, substantially similar methods to that of Binder can be employed, but using a modified neurotoxin, to treat headache, especially the ones caused by vascular disturbances, muscular tension, neuralgia and neuropathy. Pain caused by muscle spasm can also be treated by an administration of a modified neurotoxin. For example, a botulinum type E fused with a leucine-based motif, preferably at the carboxyl terminal of the botulinum type E light chain, can be administered intramuscularly at the pain/spasm location to alleviate pain.

Furthermore, a modified neurotoxin can be administered to a mammal to treat pain that is not associated with a muscular disorder, such as spasm. In one broad embodiment, methods of the present invention to treat non-spasm related pain include central administration or peripheral administration of the modified neurotoxin.

For example, Foster et al. in U.S. Patent No. 5,989,545 discloses that a botulinum toxin conjugated with a targeting moiety can be administered centrally (intrathecally) to alleviate pain. The disclosures of Foster et al. are incorporated in its entirety by reference herein. In one embodiment, substantially

similar methods to that of Foster et al. can be employed, but using the modified neurotoxin according to this invention, to treat pain. The pain to be treated can be an acute pain, or preferably, chronic
5 pain.

An acute or chronic pain that is not associated with a muscle spasm can also be alleviated with a local, peripheral administration of the modified
10 neurotoxin to an actual or a perceived pain location on the mammal. In one embodiment, the modified neurotoxin is administered subcutaneously at or near the location of pain, for example, at or near a cut. In another embodiment, the modified neurotoxin is
15 administered intramuscularly at or near the location of pain, for example, at or near a bruise location on the mammal. In another embodiment, the modified neurotoxin is injected directly into a joint of a mammal, for treating or alleviating pain caused by
20 arthritic conditions. Also, frequent repeated injection or infusion of the modified neurotoxin to a peripheral pain location is within the scope of the present invention. However, given the long lasting therapeutic effects of the present invention,
25 frequent injection or infusion of the neurotoxin can not be necessary. For example, practice of the present invention can provide an analgesic effect, per injection, for 2 months or longer, for example 27
30 months, in humans.

Without wishing to limit the invention to any mechanism or theory of operation, it is believed that when the modified neurotoxin is administered locally to a peripheral location, it inhibits the release of
5 Neuro-substances, for example substance P, from the peripheral primary sensory terminal by inhibiting SNARE-dependent exocytosis. Since the release of substance P by the peripheral primary sensory terminal can cause or at least amplify pain
10 transmission process, inhibition of its release at the peripheral primary sensory terminal will dampen the transmission of pain signals from reaching the brain.

15 In addition to having pharmacologic actions at the peripheral location, the modified neurotoxin of the present invention can also have inhibitory effects in the central nervous system, upon direct intrathecal administration, as set forth in U.S.
20 patent 6,113,915, or upon peripheral administration, where presumably the modified toxin acts through retrograde transport via a primary sensory afferent. This hypothesis of retrograde axonal transport is supported by published data which shows that
25 botulinum type A can be retrograde transported to the dorsal horn when the neurotoxin is injected peripherally. Thus, work by Weigand et al, Nauny-Schmiedeberg's Arch. Pharmacol. 1976; 292, 161-165, and Habermann, Nauny-Schmiedeberg's Arch. Pharmacol.
30 1974; 281, 47-56, showed that botulinum toxin is able to ascend to the spinal area by retrograde transport.

As such, a modified neurotoxin, for example botulinum type A with one or more amino acids mutated from the leucine-based motif, injected at a peripheral location, for example intramuscularly, can be
5 expected to be retrograde transported from the peripheral primary sensory terminal to a central region.

The amount of the modified neurotoxin
10 administered can vary widely according to the particular disorder being treated, its severity and other various patient variables including size, weight, age, and responsiveness to therapy. Generally, the dose of modified neurotoxin to be
15 administered will vary with the age, presenting condition and weight of the mammal, preferably a human, to be treated. The potency of the modified neurotoxin will also be considered.

20 Assuming a potency (for a botulinum toxin type A) which is substantially equivalent to $LD_{50} = 2,730$ U in a human patient and an average person is 75kg, a lethal dose (for a botulinum toxin type A) would be about 36 U/kg of a modified neurotoxin. Therefore,
25 when a modified neurotoxin with such an LD_{50} is administered, it would be appropriate to administer less than 36 U/kg of the modified neurotoxin into human subjects. Preferably, about 0.01 U/kg to 30 U/kg of the modified neurotoxin is administered.
30 Mor preferably, about 1 U/kg to about 15 U/kg of the modified neurotoxin is administered. Even more

preferably, about 5 U/kg to about 10 U/kg modified neurotoxin is administered. Generally, the modified neurotoxin will be administered as a composition at a dosage that is proportionally equivalent to about 2.5 cc/100 U. Those of ordinary skill in the art will know, or can readily ascertain, how to adjust these dosages for neurotoxin of greater or lesser potency. It is known that botulinum toxin type B can be administered at a level about fifty times higher than that used for a botulinum toxin type A for similar therapeutic effect. Thus, the units amounts set forth above can be multiplied by a factor of about fifty for a botulinum toxin type B..

Although examples of routes of administration and dosages are provided, the appropriate route of administration and dosage are generally determined on a case by case basis by the attending physician. Such determinations are routine to one of ordinary skill in the art (see for example, *Harrison's Principles of Internal Medicine* (1998), edited by Anthony Fauci et al., 14th edition, published by McGraw Hill). For example, the route and dosage for administration of a modified neurotoxin according to the present disclosed invention can be selected based upon criteria such as the solubility characteristics of the modified neurotoxin chosen as well as the types of disorder being treated.

The modified neurotoxin can be produced by chemically linking the leucine-based motif to a

neurotoxin using conventional chemical methods well known in the art. For example, botulinum type E can be obtained by establishing and growing cultures of *Clostridium botulinum* in a fermenter, and then
5 harvesting and purifying the fermented mixture in accordance with known procedures.

The modified neurotoxin can also be produced by recombinant techniques. Recombinant techniques are
10 preferable for producing a neurotoxin having amino acid sequence regions from different *Clostridial* species or having modified amino acid sequence regions. Also, the recombinant technique is preferable in producing botulinum type A with the
15 leucine-based motif being modified by deletion. The technique includes steps of obtaining genetic materials from natural sources, or synthetic sources, which have codes for a cellular binding moiety, an amino acid sequence effective to translocate the
20 neurotoxin or a part thereof, and an amino acid sequence having therapeutic activity when released into a cytoplasm of a target cell, preferably a neuron. In a preferred embodiment, the genetic materials have codes for the biological persistence
25 enhancing component, preferably the leucine-based motif, the H_C, the H_N and the light chain of the *Clostridial* neurotoxins and fragments thereof. The genetic constructs are incorporated into host cells for amplification by first fusing the genetic
30 constructs with a cloning vectors, such as phag s or plasmids. Then the cloning vectors are inserted into

a host, for example, Clostridium sp., E. coli or other prokaryotes, yeast, insect cell line or mammalian cell lines. Following the expressions of the recombinant genes in host cells, the resultant
5 proteins can be isolated using conventional techniques.

There are many advantages to producing these modified neurotoxins recombinantly. For example, to
10 form a modified neurotoxin, a modifying fragment, or component must be attached or inserted into a neurotoxin. The production of neurotoxin from anaerobic Clostridium cultures is a cumbersome and time-consuming process including a multi-step
15 purification protocol involving several protein precipitation steps and either prolonged and repeated crystallization of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure
20 must be performed under strict containment (BL-3). During the fermentation process, the folded single-chain neurotoxins are activated by endogenous Clostridial proteases through a process termed nicking to create a dichain. Sometimes, the process
25 of nicking involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the intrachain disulfide bond.

30

The nicked neurotoxin is much more active than the unnicked form. The amount and precise location of nicking varies with the serotypes of the bacteria producing the toxin. The differences in single-chain neurotoxin activation and, hence, the yield of nicked toxin, are due to variations in the serotype and amounts of proteolytic activity produced by a given strain. For example, greater than 99% of *Clostridial botulinum* serotype A single-chain neurotoxin is activated by the Hall A *Clostridial botulinum* strain, whereas serotype B and E strains produce toxins with lower amounts of activation (0 to 75% depending upon the fermentation time). Thus, the high toxicity of the mature neurotoxin plays a major part in the commercial manufacture of neurotoxins as therapeutic agents.

The degree of activation of engineered *Clostridial* toxins is, therefore, an important consideration for manufacture of these materials. It would be a major advantage if neurotoxins such as botulinum toxin and tetanus toxin could be expressed, recombinantly, in high yield in rapidly-growing bacteria (such as heterologous *E. coli* cells) as relatively non-toxic single-chains (or single chains having reduced toxic activity) which are safe, easy to isolate and simple to convert to the fully-active form.

With safety being a prime concern, previous work has concentrated on the expression in *E. coli*

and purification of individual H and light chains of tetanus and botulinum toxins; these isolated chains are, by themselves, non-toxic; see Li et al., *Biochemistry* 33:7014-7020 (1994); Zhou et al., *Biochemistry* 34:15175-15181 (1995), hereby incorporated by reference herein. Following the separate production of these peptide chains and under strictly controlled conditions the H and light chains can be combined by oxidative disulphide linkage to form the neuromuscular di-chains.

EXAMPLES

The following non-limiting examples provide those of ordinary skill in the art with specific preferred methods to treat non-spasm related pain within the scope of the present invention and are not intended to limit the scope of the invention.

Example 1

Treatment of Pain Associated with Muscle Disorder

An unfortunate 36 year old woman has a 15 year history of temporomandibular joint disease and chronic pain along the masseter and temporalis muscles. Fifteen years prior to evaluation she noted increased immobility of the jaw associated with pain and jaw opening and closing and tenderness along each side of her face. The left side is originally thought to be worse than the right. She is diagnosed as having temporomandibular joint (TMJ) dysfunction with subluxation of the joint and is treated with

surgical orthoplasty meniscus ctomy and condyle resection.

5 She continues to have difficulty with opening and closing her jaw after the surgical procedures and for this reason, several years later, a surgical procedure to replace prosthetic joints on both sides is performed. After the surgical procedure progressive spasms and deviation of the jaw ensues.

10 Further surgical revision is performed subsequent to the original operation to correct prosthetic joint loosening. The jaw continues to exhibit considerable pain and immobility after these surgical procedures. The TMJ remained tender as well as the muscle itself.

15 There are tender points over the temporomandibular joint as well as increased tone in the entire muscle. She is diagnosed as having post-surgical myofascial pain syndrome and is injected with the modified neurotoxin into the masseter and temporalis muscles;

20 the modified neurotoxin is botulinum type E comprising a leucine-based motif. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician.

25

Several days after the injections she noted substantial improvement in her pain and reports that her jaw feels looser. This gradually improves over a 2 to 3 week period in which she notes increased

30 ability to open the jaw and diminishing pain. The patient states that the pain is better than at any

time in the last 4 years. Th improved condition persists for up to 27 months after the original injection of the modified neurotoxin.

5

Example 2Treatment of Pain Subsequent to Spinal Cord Injury

A patient, age 39, experiencing pain subsequent to spinal cord injury is treated by intrathecal administration, for example, by spinal tap or by
10 catherization (for infusion) to the spinal cord, with the modified neurotoxin; the modified neurotoxin is botulinum type E comprising a leucine-based motif. The particular toxin dose and site of injection, as well as the frequency of toxin administrations,
15 depend upon a variety of factors within the skill of the treating physician, as previously set forth. Within about 1 to about 7 days after the modified neurotoxin administration, the patient's pain is substantially reduced. The pain alleviation persists
20 for up to 27 months.

Example 3Peripheral Administration of a Modified Neurotoxin to
Treat "Shoulder-Hand Syndrome"

25 Pain in the shoulder, arm, and hand can develop, with muscular dystrophy, osteoporosis and fixation of joints. While most common after coronary insufficiency, this syndrome can occur with cervical osteoarthritis or localized shoulder disease, or

after any prolonged illness that requires the patient to remain in bed.

A 46 year old woman presents a shoulder-hand
5 syndrome type pain. The pain is particularly
localized at the deltoid region. The patient is
treated by a bolus injection of a modified neurotoxin
subcutaneously to the shoulder; preferably the
modified neurotoxin is botulinum type E comprising a
10 leucine-based motif. The modified neurotoxin can
also be, for example, modified botulinum type A, B,
C1, C2, D, E, F or G which comprise a leucine-based
motif. The particular dose as well as the frequency
of administrations depends upon a variety of factors
15 within the skill of the treating physician, as
previously set forth. Within 1-7 days after modified
neurotoxin administration the patient's pain is
substantially alleviated. The duration of the pain
alleviation is from about 7 to about 27 months.

20

Example 4

Peripheral Administration of a Modified Neurotoxin to Treat Posttherapeutic Neuralgia

Posttherapeutic neuralgia is one of the most
25 intractable of chronic pain problems. Patients
suffering this excruciatingly painful process often
are elderly, have debilitating disease, and are not
suitable for major interventional procedures. The
diagnosis is readily made by the appearance of the
30 heal d lesions of herpes and by the patient's

nistory. The pain is intense and emotionally distressing. Posttherapeutic neuralgia can occur anywhere, but is most often in the thorax.

5 A 76 year old man presents a posttherapeutic type pain. The pain is localized to the abdomen region. The patient is treated by a bolus injection of a modified neurotoxin intradermally to the abdomen; the modified neurotoxin is, for example, botulinum type
10 A, B, C1, C2, D, E, F and/or G. The modified neurotoxin comprises a leucine-based motif and/or additional tyrosine-based motifs. The particular dose as well as the frequency of administration depends upon a variety of factors within the skill of
15 the treating physician, as previously set forth. Within 1-7 days after modified neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

20

Example 5

Peripheral Administration of a Modified Neurotoxin to Treat Nasopharyngeal Tumor Pain

25 These tumors, most often squamous cell carcinomas, are usually in the fossa of Rosenmuller and can invade the base of the skull. Pain in the face is common. It is constant, dull-aching in nature.

A 35 year old man presents a nasopharyngeal tumor type pain. Pain is found at the lower left cheek. The patient is treated by a bolus injection of a modified neurotoxin intramuscularly to the
5 cheek, preferably the modified neurotoxin is botulinum type A, B, C1, C2, D, E, F or G comprising additional biological persistence enhancing amino acid derivatives, for example, tyrosine phosphorylations. The particular dose as well as the
10 frequency of administrations depends upon a variety of factors within the skill of the treating physician. Within 1-7 days after modified neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is
15 from about 7 to about 27 months.

Example 6

Peripheral Administration of a Modified Neurotoxin to Treat Inflammatory Pain

20

A patient, age 45, presents an inflammatory pain in the chest region. The patient is treated by a bolus injection of a modified neurotoxin intramuscularly to the chest, preferably the modified
25 neurotoxin is botulinum type A, B, C1, C2, D, E, F or G comprising additional tyrosine-based motifs. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician, as
30 previously set forth. Within 1-7 days after modified

neurotoxin administration the patient's pain is substantially alleviated. The duration of the pain alleviation is from about 7 to about 27 months.

5

Example 7

Treatment of Excessive Sweating

A male, age 65, with excessive unilateral sweating is treated by administering a modified neurotoxin. The dose and frequency of administration depends upon degree of desired effect. Preferably, the modified neurotoxin is botulinum type A, B, C1, C2, D, E, F and/or G. The modified neurotoxins comprise a leucine-based motif. The administration is to the gland nerve plexus, ganglion, spinal cord or central nervous system. The specific site of administration is to be determined by the physician's knowledge of the anatomy and physiology of the target glands and secretory cells. In addition, the appropriate spinal cord level or brain area can be injected with the toxin. The cessation of excessive sweating after the modified neurotoxin treatment is up to 27 months.

20

15

10

Example 8

Post Surgical Treatments

25

A female, age 22, presents a torn shoulder tendon and undergoes orthopedic surgery to repair the tendon. After the surgery, the patient is administered intramuscularly with a modified neurotoxin to the shoulder. The modified neurotoxin

30

can botulinum type A, B, C, D, E, F, and/or G wherein one or more amino acids of a biological persistence enhancing component are deleted from the toxin. For example, one or more leucine residues can be deleted from and/or mutated from the leucine-based motif in botulinum toxin serotype A. Alternatively, one or more amino acids of the leucine-based motif can be substituted for other amino acids. For example, the two leucines in the leucine-based motif can be substituted for alanines. The particular dose as well as the frequency of administrations depends upon a variety of factors within the skill of the treating physician. The specific site of administration is to be determined by the physician's knowledge of the anatomy and physiology of the muscles. The administered modified neurotoxin reduces movement of the arm to facilitate the recovery from the surgery. The effect of the modified neurotoxin is for about five weeks or less.

20

Example 9

Cloning, Expression and Purification of the Botulinum Neurotoxin Light Chain Gene

This example describes methods to clone and express a DNA nucleotide sequence encoding a botulinum toxin light chain and purify the resulting protein product. A DNA sequence encoding the botulinum toxin light chain can be amplified by PCR protocols which employ synthetic oligonucleotides having sequences corresponding to the 5' and 3' end regions of the light chain gene. Design of th

primers can allow for the introduction of restriction sites, for example, Stu I and EcoR I restriction sites into the 5' and 3' ends of the botulinum toxin light chain gene PCR product. These restriction
5 sites can be subsequently used to facilitate unidirectional subcloning of the amplification products. Additionally, these primers can introduce a stop codon at the C-terminus of the light chain coding sequence. Chromosomal DNA from *C. botulinum*,
10 for example, strain HallA, can serve as a template in the amplification reaction.

The PCR amplification can be performed in a 0.1 mL volume containing 10 mM Tris-HCl (pH 8.3), 50 mM
15 KCl, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 50 pmol of each primer, 200 ng of genomic DNA and 2.5 units of Taq DNA polymerase. The reaction mixture can be subjected to 35 cycles of denaturation (1 minute at 94° C), annealing (2
20 minutes at 55°C) and polymerization (2 minutes at 72°C). Finally, the reaction can be extended for an additional 5 minutes at 72°C.

The PCR amplification product can be digested
25 with for example, Stu I and EcoR I, to release the light chain encoding, cloned, PCR DNA fragment. This fragment can then be purified by, for example, agarose gel electrophoresis, and ligated into, for example, a Sma I and EcoR I digested pBluescript II
30 SK phagemid. Bacterial transformants, for example, E coli, harboring this recombinant phagemid can be identified by standard procedures, such as blue/white

screening. Clones comprising the light chain encoding DNA can be identified by DNA sequence analysis performed by standard methods. The cloned sequences can be confirmed by comparing the cloned sequences to published sequences for botulinum light chains, for example, Binz, et al., in *J. Biol. Chem.* 265, 9153 (1990), Thompson et al., in *Eur. J. Biochem.* 189, 73 (1990) and Minton, *Clostridial Neurotoxins, The Molecular Pathogenesis of Tetanus and Botulism* p. 161-191, Edited by C. Motecucco (1995).

The light chain can be subcloned into an expression vector, for example, pMal-P2. pMal-P2 harbors the *malE* gene encoding MBP (maltose binding protein) which is controlled by a strongly inducible promoter, P_{tac} .

To verify expression of the botulinum toxin light chain, a well isolated bacterial colony harboring the light chain gene containing pMal-P2 can be used to inoculate L-broth containing 0.1 mg/ml ampicillin and 2% (w/v) glucose, and grown overnight with shaking at 30°C. The overnight cultures can be diluted 1:10 into fresh L-broth containing 0.1 mg/ml of ampicillin and incubated for 2 hours. Fusion protein expression can be induced by addition of IPTG to a final concentration of 0.1 mM. After an additional 4 hour incubation at 30°C, bacteria can be collected by centrifugation at 6,000 x g for 10 minutes.

A small-scale SDS-PAGE analysis can confirm the presence of a 90 kDa protein band in samples derived from IPTG-induced bacteria. This MW would be consistent with the predicted size of a fusion protein having MBP (~ 40 kDa) and botulinum toxin light chain (~ 50 kDa) components.

The presence of the desired fusion proteins in IPTG-induced bacterial extracts can be confirmed by western blotting using the polyclonal anti-L chain probe described by Cenci di Bello et al., in *Eur. J. Biochem.* 219, 161 (1993). Reactive bands on PVDF membranes (Pharmacia; Milton Keynes, UK) can be visualized using an anti-rabbit immunoglobulin conjugated to horseradish peroxidase (BioRad; Hemel Hempstead, UK) and the ECL detection system (Amersham, UK). Western blotting results typically confirm the presence of the dominant fusion protein together with several faint bands corresponding to proteins of lower MW than the fully sized fusion protein. This observation suggests that limited degradation of the fusion protein occurred in the bacteria or during the isolation procedure.

To produce the subcloned light chain, pellets from 1 liter cultures of bacteria expressing the wild-type Botulinum neurotoxin light chain proteins can be resuspended in column buffer [10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EGTA and 1 mM DTT] containing 1mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM benzamidine, and lysed by sonication. The lysates can be cleared by centrifugation at 15,000 x

g for 15 minutes at 4°C. Supernatants can be applied to an amylose affinity column [2x10 cm, 30 ml resin] (New England BioLabs; Hitchin, UK). Unbound proteins can be washed from the resin with column buffer until the eluate is free of protein as judged by a stable absorbance reading at 280 nm. The bound MBP-L chain fusion protein can be subsequently eluted with column buffer containing 10 mM maltose. Fractions containing the fusion protein can be pooled and dialyzed against 20 mM Tris-HCl (pH 8.0) supplemented with 150 mM NaCl, 2 mM, CaCl₂ and 1 mM DTT for 72 hours at 4°C.

The MBP-L chain fusion proteins can be purified after release from the host bacteria. Release from the bacteria can be accomplished by enzymatically degrading or mechanically disrupting the bacterial cell membrane. Amylose affinity chromatography can be used for purification. Recombinant wild-type or mutant light chains can be separated from the sugar binding domains of the fusion proteins by site-specific cleavage with Factor Xa. This cleavage procedure typically yields free MBP, free light chains and a small amount of uncleaved fusion protein. While the resulting light chains present in such mixtures can be shown to possess the desired activities, an additional purification step can be employed. For example, the mixture of cleavage products can be applied to a second amylose affinity column which binds both the MBP and uncleaved fusion protein. Free light chains can be isolated in the flow through fraction.

Example 10Reconstitution of Native light chain, Recombinant
5 Wild-Type light chain with Purified Heavy chain

Native heavy and light chains can be dissociated from a BoNT with 2 M urea, reduced with 100 mM DTT and then purified according to established
10 chromatographic procedures. For example, Kozaki et al. (1981, *Japan J. Med. Sci. Biol.* 34, 61) and Maisey et al. (1988, *Eur. J. Biochem.* 177, 683). A purified heavy chain can be combined with an equimolar amount of either native light chain or a
15 recombinant light chain. Reconstitution can be carried out by dialyzing the samples against a buffer consisting of 25 mM Tris (pH 8.0), 50 μ M zinc acetate and 150 mM NaCl over 4 days at 4°C. Following dialysis, the association of the recombinant light
20 chain and native heavy chain to form disulfide linked 150 kDa dichains is monitored by SDS-PAGE and/or quantified by densitometric scanning.

Example 11Production of a Modified Neurotoxin with an Enhanced
25 Biological Persistence

A modified neurotoxin can be produced by
30 employing recombinant techniques in conjunction with conventional chemical techniques.

A neurotoxin chain, for example a botulinum light chain that is to be fused with a biological persistence enhancing component to form a modified neurotoxin can be produced recombinantly and purified
5 as described in example 9.

The recombinant neurotoxin chain derived from the recombinant techniques can be covalently fused with (or coupled to) a biological persistence
10 enhancing component, for example a leucine-based motif, a tyrosine-based motif and/or an amino acid derivative. Peptide sequences comprising biological persistence enhancing components can be synthesized by standard t-Boc/Fmoc technologies in solution or
15 solid phase as is known to those skilled in the art. Similar synthesis techniques are also covered by the scope of this invention, for example, methodologies employed in Milton et al. (1992, *Biochemistry* 31, 8799-8809) and Swain et al. (1993, *Peptide Research*
20 6, 147-154). One or more synthesized biological persistence enhancing components can be fused to the light chain of botulinum type A, B, C1, C2, D, E, F or G at, for example, the carboxyl terminal end of the toxin. The fusion of the biological persistence
25 enhancing components is achieved by chemical coupling using reagents and techniques known to those skilled in the art, for example PDPH/EDAC and Traut's reagent chemistry.

30 Alternatively, a modified neurotoxin can be produced recombinantly without the step of fusing the biological persistence enhancing component to a

r combinant botulinum toxin chain. For example, a recombinant neurotoxin chain, for example, a botulinum light chain, derived from the recombinant techniques of example 9 can be produced with a biological persistence enhancing component, for example a leucine-based motif, a tyrosine-based motif and/or an amino acid derivative. For example, one or more DNA sequences encoding biological persistence enhancing components can be added to the DNA sequence encoding the light chain of botulinum type A, B, C1, C2, D, E, F or G. This addition can be done by any number of methods used for site directed mutagenesis which are familiar to those skilled in the art.

The recombinant modified light chain containing the fused or added biological persistence enhancing component can be reconstituted with a heavy chain of a neurotoxin by the method described in example 10 thereby producing a complete modified neurotoxin.

The modified neurotoxins produced according to this example have an enhanced biological persistence. Preferably, the biological persistence is enhanced by about 20% to about 300% relative to an identical neurotoxin without the additional biological persistence enhancing component(s).

Example 12

Production of a Modified Neurotoxin with a Reduced Biological Persistence

A modified neurotoxin with a reduced biological persistence can be produced by employing recombinant techniques. For example, a botulinum light chain derived from the recombinant techniques of example 9
5 can be produced without a biological persistence enhancing component. For example, one or more leucine-based motifs, tyrosine-based motifs and/or amino acid derivatives can be mutated. For example, one or more DNA sequences encoding biological
10 persistence enhancing components can be removed from the DNA sequence encoding the light chain of botulinum type A, B, C1, C2, D, E, F or G. For example, the DNA sequence encoding the leucine based motif can be removed from the DNA sequence encoding
15 botulinum type A light chain. Removal of the DNA sequences can be done by any number of methods familiar to those skilled in the art.

The recombinant modified light chain with the
20 deleted biological persistence enhancing component can be reconstituted with a heavy chain of a neurotoxin by the method described in example 10 thereby producing a complete modified neurotoxin.

25 The modified neurotoxin produced according to this example has a reduced biological persistence. Preferably, the biological persistence is reduced by about 20% to about 300% relative to an identical neurotoxin, for example botulinum type A, with the
30 leucine-based motif.

Although the present invention has been described in detail with regard to certain preferred methods, other embodiments, versions, and modifications within the scope of the present invention are possible. For example, a wide variety of modified neurotoxins can be effectively used in the methods of the present invention in place of Clostridial neurotoxins. Also, the corresponding genetic codes, i.e. DNA sequence, to the modified neurotoxins are also considered to be part of this invention. Additionally, the present invention includes peripheral administration methods wherein two or more modified neurotoxins, for example botulinum type E with a fused leucine-based motif, and botulinum type B comprising a leucine-based motif, are administered concurrently or consecutively. While this invention has been described with respect to various specific examples and embodiments, it is to be understood that the invention is not limited thereto and that it can be variously practiced with the scope of the following claims.

Example 13

Production of a Modified Neurotoxin with a Reduced Biological Persistence

25

Localization to the cellular membrane is likely a key factor in determining the biological persistence of botulinum toxins. This is because localization to a cell membrane can protect the localized protein from inter-cellular protein degrading complexes.

It is well known and generally accepted that the biological persistence of botulinum type B neurotoxin is shorter than the biological persistence of botulinum type A neurotoxin. In this work, it was demonstrated that when the botulinum toxin type A light chain is truncated, which comprises removing the leucine-based motif, the light chain substantially loses its ability to localize to the cellular membrane in its characteristic pattern. In fact, truncated type A light chain localizes to the cellular membrane in a pattern similar to that of botulinum toxin type B light chain.

Therefore, it can be hypothesized that truncated botulinum type A has a reduced biological persistence and/or a reduced biological activity similar to that of botulinum toxin type B.

Example 14

Production of a Modified Neurotoxin with an Altered Biological Persistence

Localization to the cellular membrane is likely a key factor in determining the biological persistence of botulinum toxins. This is because localization to a cell membrane can protect the localized protein from inter-cellular protein degrading complexes.

In this work, it was demonstrated that when the botulinum toxin type A light chain is mutated, changing the two leucines at positions 427 and 428 to alanines (Fig. 3), the light chain substantially

2885CIP)

loses its ability to localize to the cellular membrane in its characteristic pattern.

From this data it can be concluded that the mutated botulinum type A has an altered biological persistence.

Example 15

In Vitro Cleavage of SNAP 25 by Truncated LC/A

10 As illustrated by Fig 9, an in vitro ELISA assay was carried out by the inventors demonstrating that a truncated LC/A in vitro cleaves SNAP-25 substrate less efficiently than does non-truncated LC/A. The data displayed is not a measure of inhibition of
15 ~~exocytosis but a measure of the in vitro formation of~~ SNAP-25 cleavage. The assay was carried out as follows:

Materials:

- 20 BirA-SNAP25₁₂₈₋₂₀₆ - this is a recombinant substrate for LC/A, consisting of a BirA signal sequence fused to the N-terminus of residues 128 - 206 of SNAP25. This fusion construct was produced in E. coli and the BirA signal sequence was biotinylated by the E. coli.
- 25 Microtiter plates were coated with streptavidin. The toxin used was BoNT/A complex or LC/A constructs. The primary antibody was anti-SNAP25₁₉₇ antibody. This antibody recognizes the C-terminus of SNAP25 following cleavage by Type A toxin (BirA-SNAP25₁₂₈₋₁₉₇).
- 30 The secondary antibody was goat, anti-rabbit IgG conjugated to horseradish peroxidase. The ImmunoPure

2885CIP)
TMB substrate was from Pierce, a colorimetric
substrate for horseradish peroxidase. The antibody
that recognizes the cleaved product SNAP25₁₋₉₇ is
specific for that cleaved product and does not
5 recognize the full length uncleaved substrate
SNAP25₁₋₂₀₆.

Method:

10 BixA-SNAP25₁₋₂₀₆ was bound to streptavidin on a
microtiter plate. To the plates were added serial
dilutions of BoNT/A 900 kDa complex, His6-S-
nativeLC/A, or His6-S-truncLC/A-His6. All toxin
samples were pre-incubated with DTT (this is not
15 required for the LC/A constructs, but they were
treated the same as the BoNT/A complex). The toxin
samples were incubated with the substrate for 90
minutes at 37 °C. The toxin was removed and the bound
substrate was incubated with anti-SNAP25₁₋₉₇ antibody.
20 Unbound antibody was washed away and the plates were
then incubated with the secondary antibody (anti-
rabbit IgG conjugated to horseradish peroxidase).
Unbound antibody was again washed away and a
colorimetric assay for horseradish peroxidase was
25 performed. The assay was quantified by reading the
absorbance at 450 nm.

In other work by the inventors disclosed herein the
light chain constructs that were expressed in the PC-
30 12 cells were expressed directly in the PC-12 cells
and do not contain any tags. The light chain
constructs that have been expressed from *E. coli* for

these in vitro assays contain affinity tags for purification purposes (these tags are not present on the proteins expressed in the PC-12 cells, as disclosed herein). The LC/A expressed in PC12 was the fusion protein GFP-LC/A. Between the GFP and the LC/A there is a set of Gly to separate both proteins.

An explanation of the various constructs follows:
Complex (red in the graph) this is BoNT/A 900 kDa complex isolated from C. botulinum

Truncated LC/A - construct lacking 8 amino acids at the N-terminus and 22 amino acids at the C-terminus. However, this construct does contain a 6-histidine and an S-tag at the N-terminus as well as a 6-histidine tag at the C-terminus.

Dialyzed Truncated LC/A - same as Truncated LC/A, but imidazole resulting from the purification has been removed.

Full LC/A (Dark green in graph) - native LC/A construct (full-length), but containing the N-terminal 6-histidine and S-tag. Does not have the C-terminal 6-histidine.

Dialyzed Full LC/A (Light green in graph) - Same as Full LC/A, but imidazole resulting from the purification has been removed.

To graphically depict these differences, Fig 10 shows the very N-terminus and the very C-terminus of these

constructs (the middle portion of the LC/A proteins is not shown). What is referred to as Wildtype corresponds to the native LC/A that the inventors had expressed directly in the PC-12 cells (this is
5 construct that the inventors demonstrated activity with via Western blot analysis of the cleaved SNAP25 product). Truncated LC/A is the truncated light chain containing the His and S-tags. N-His-LC/A is what was referred to as Full LC/A in Fig. 9.

10

What is claimed is:

1. A modified neurotoxin comprising:
 - a neurotoxin including a structural
5 modification, wherein said structural modification is
effective to alter a biological persistence of said
modified neurotoxin relative to an identical
neurotoxin without said structural modification, and
wherein said modified neurotoxin is structurally
10 different from a naturally existing neurotoxin.
2. The modified neurotoxin of claim 1, wherein said
structural modification is effective to enhance a
biological persistence of said modified neurotoxin.
15
3. The modified neurotoxin of claim 1 wherein said
biological persistence of said modified neurotoxin is
reduced relative to an identical neurotoxin without
said structural modification.
20
4. The modified neurotoxin of claim 1 wherein said
structural modification comprises 1 to about 22 amino
acids.
- 25 5. The modified neurotoxin of claim 1 wherein said
structural modification comprises an amino acid, said
amino acid comprising an R group of 1 to about 12
carbon atoms.
- 30 6. The modified neurotoxin of claim 1 wherein said
structural modification comprises a leucine-based
motif (SEQ ID NO: 1).

7. The modified neurotoxin of claim 1 where in said structural modification comprises a tyrosine-based motif.

5

8. The modified neurotoxin of claim 1 wherein said structural modification comprises an amino acid sequence of a botulinum type A light chain and of an amino acid sequence of a type B light chain.

10

9. The modified neurotoxin of claim 8 wherein said structural modification comprises the amino acid sequence KAFK.

15

10. The modified neurotoxin of claim 8 wherein said structural modification comprises the amino acid sequence YYD in combination with the amino acid sequence YYL in combination with the amino acid sequence T.

20

11. The modified neurotoxin of claim 1 wherein said structural modification comprises an amino acid derivative.

25

12. The modified neurotoxin of claim 1 wherein said neurotoxin is selected from the group consisting of botulinum toxin type A, B, C₁, C₂, D, E, F and G.

30

13. The modified neurotoxin of claim 1 wherein said neurotoxin is botulinum toxin type A.

14. A modified neurotoxin comprising a neurotoxin including a structural modification, wherein said neurotoxin comprises three amino acid sequence regions:

5

- a) a first region effective as a cellular binding moiety;
- b) a second region effective to translocate a modified neurotoxin or a part thereof across an endosome membrane; and
- c) a third region effective to inhibit exocytosis when released into a cytoplasm of a target cell,

10

15

wherein at least one of said first, said second and said third regions is substantially derived from a Clostridial neurotoxin, said third region includes said structural modification, a modified neurotoxin is structurally different from a naturally existing neurotoxin, and said structural modification is effective to alter a biological persistence of said modified neurotoxin relative to an identical neurotoxin without said structural modification.

25

15. The modified neurotoxin of claim 14, wherein said neurotoxin is a member selected from a group consisting of botulinum toxin serotypes A, B, C₁, C₂, D, E, F, G, tetanus toxin and mixtures thereof.

30

16. The modified neurotoxin of claim 14, wherein said neurotoxin is a member selected from a group

consisting of botulinum toxin serotypes A, B, C₁, C₂,
D, E, F and G.

17. The modified neurotoxin of claim 14, wherein
5 said neurotoxin comprises botulinum toxin serotypes
A.

18. The modified neurotoxin of claim 14, wherein
said third region is derived from botulinum toxin
10 serotype A.

19. The modified neurotoxin of claim 14, wherein
said third region is not derived from botulinum toxin
serotype A.

15 20. The modified neurotoxin of claim 14, wherein
said structural modification includes a biological
persistence enhancing component effective to enhance
said biological persistence of said modified
20 neurotoxin.

21. The modified neurotoxin of claim 20, wherein
said biological persistence enhancing component
comprises a leucine-based motif of SEQ ID NO: 1.

25 22. The modified neurotoxin of claim 20, wherein
said leucine-based motif comprises a run of seven
amino acids, wherein said run comprises a quintet of
amino acids and a duplet of amino acids, wherein said
30 quintet of amino acids defines the amino terminal end
of said leucine-based motif and said duplet of amino

acids defines the carboxyl end of said leucine-based motif.

23. The modified neurotoxin of claim 22, wherein
5 said quintet of amino acids comprises an acidic amino acid, wherein said acidic amino acid is selected from a group consisting of a glutamate and an aspartate.

24. The modified neurotoxin of claim 22, wherein
10 said quintet of amino acids comprises a hydroxyl containing amino acid, wherein said hydroxyl containing amino acid is selected from the group consisting of a serine, a threonine and a tyrosine.

15 25. The modified neurotoxin of claim 24, wherein
said hydroxyl containing amino acid can be phosphorylated.

26. The modified neurotoxin of claim 22, wherein
20 said duplet of amino acids comprises at least one amino acid, wherein said amino acid is selected from the group consisting of leucine, isoleucine, methionine, alanine, phenylalanine, tryptophan, valine and tyrosine.

25
27. The modified neurotoxin of claim 22, wherein
said duplet of amino acids is selected from a group
consisting of leucine-leucine, leucine-isoleucine,
isoleucine-leucine, isoleucine-isoleucine and leucine
30 -methionine.

28. The modified neurotoxin of claim 21, wherein said leucine-based motif comprises an amino acid sequence phenylalanine-glutamate-phenylalanine-tyrosine-lysine-leucine-leucine of SEQ ID NO: 1.

5

29. A modified neurotoxin wherein said modification comprises a tyrosine-based motif.

30. The modified neurotoxin of claim 29 wherein said
10 tyrosine-based motif comprises a run of four amino acids, wherein an amino acid comprising an N-terminal end of said run comprises a tyrosine residue and an amino acid comprising a C-terminal end of said run comprises a hydrophobic amino acid.

15

31. The modified neurotoxin of claim 14, wherein said biological persistence of said modified neurotoxin is reduced relative to an identical neurotoxin without said structural modification.

20

32. The modified neurotoxin of claim 31, wherein said structural modification includes a leucine-based motif with mutation to one or more amino acids comprising said leucine-based motif of SEQ ID NO: 1.

25

33. The modified neurotoxin of claim 31, wherein said structural modification includes a tyrosine-based motif with a mutation to one or more amino acids comprising said tyrosine-based motif.

30

34. The modified neurotoxin of claim 31, wherein said structural modification comprises an amino acid

derivativ with a mutation to on or more amino acids comprising said amino acid derivative.

35. A method for enhancing the biological
5 persistence of a neurotoxin of claim 14, wherein a structural modification is fused or added to said neurotoxin.

36. The modified neurotoxin of claim 35 wherein said
10 structural modification comprises a leucine-based motif.

37. The modified neurotoxin of claim 35 wherein said
15 structural modification comprises a tyrosine-based motif.

38. The modified neurotoxin of claim 35 wherein said
structural modification comprises an amino acid
20 derivative.

39. A modified neurotoxin comprising:
a botulinum type A neurotoxin including a structural
modification, wherein said structural modification is
effective to alter a biological persistence of said
25 modified neurotoxin relative to an identical
neurotoxin without said structural modification,
wherein said structural modification comprises a
deletion of amino acids 1 to 8 and 416 to 437 from a
light chain of said neurotoxin.

30
40. A modified neurotoxin comprising:

a botulinum type A neurotoxin including a structural modification, wherein said structural modification is effective to alter a biological persistence of said modified neurotoxin relative to an identical
5 neurotoxin without said structural modification, wherein said structural modification comprises substitution of leucine at position 427 for an alanine and leucine at position 428 for an alanine in a light chain of said neurotoxin.

10

41. A method for reducing the biological persistence of a neurotoxin comprising the step of mutating an amino acid of the neurotoxin.

15 42. The method of claim 41 which comprises the step of deleting or substituting said amino acid of a leucine-based motif within the neurotoxin.

20 43. The method of claim 41 which comprises the step of deleting or substituting said amino acid from a tyrosine-based motif within the neurotoxin.

25 44. The method of claim 41 which comprises the step of deleting or substituting an amino acid derivative within the neurotoxin.

30 45. A method of treating a condition, comprising a step of administering an effective dose of a modified neurotoxin to a mammal to treat a condition, wherein said modified neurotoxin comprises a neurotoxin including a structural modification, and wherein said

structural modification is effective to alter a biological persistence of said neurotoxin.

46. The method of treating a condition of claim 45,
5 wherein said neurotoxin does not comprise a leucine-based motif.

47. The method of treating said condition of claim
46, wherein said structural modification includes a
10 biological persistence enhancing component.

48. The method of claim 47 wherein said biological
persistence enhancing component comprises a leucine-
based motif.

15

49. The method of claim 47 wherein said biological
persistence enhancing component comprises a tyrosine-
based motif.

20 50. The method of claim 47 wherein said biological
persistence enhancing component comprises an amino
acid derivative.

51. The method of treating said condition of
25 claim 45, wherein said condition comprises a
condition selected from the group consisting of a
neuromuscular disorder, an autonomic disorder and
pain.

30 52. The method of treating said condition of claim
51, wherein treatment of said neuromuscular disorder
comprises a step of locally administering an

effective amount of said modified neurotoxin to a muscle or group of muscles.

53. The method of treating said condition of claim 51, wherein treatment of said autonomic disorder comprises a step of locally administering an effective amount of said modified neurotoxin to a gland.

54. The method of treating said condition claim 51, wherein treatment of pain comprises a step of administering an effective amount of said modified neurotoxin to a site of pain.

55. The method of treating said condition of claim 51, wherein treatment of pain comprises a step of administering an effective amount of said modified neurotoxin to a spinal cord.

56. The method of treating said condition of claim 45, wherein said condition is selected from the group consisting of spasmodic dysphonia, laryngeal dystonia, oromandibular dysphonia, lingual dystonia, cervical dystonia, focal hand dystonia, blepharospasm, strabismus, hemifacial spasm, eyelid disorder, cerebral palsy, focal spasticity, spasmodic colitis, neurogenic bladder, anismus, limb spasticity, tics, tremors, bruxism, anal fissure, achalasia, dysphagia, lacrimation, hyperhidrosis, excessive salivation, excessive gastrointestinal secretions, pain from muscle spasms, headache pain, brow furrows and skin wrinkles.

57. A modified neurotoxin comprising:

5 a neurotoxin including a structural modification, wherein said structural modification is effective to alter a biological activity of said modified neurotoxin relative to an identical neurotoxin without said structural modification, and wherein said modified neurotoxin is structurally different from a naturally existing neurotoxin.

10

58. The modified neurotoxin of claim 57, wherein said structural modification is effective to reduce an exocytosis from a target cell by more than the amount of the exocytosis reduced from the target cell by an identical neurotoxin without said structural modification.

15

59. The modified neurotoxin of claim 57, wherein said structural modification is effective to reduce an exocytosis from a target cell by less than the amount of the exocytosis reduced from the cell by an identical neurotoxin without said structural modification.

20

25 60. The modified neurotoxin of claim 58 or claim 59 wherein the exocytosis is exocytosis of a neurotransmitter.

25

61. The modified neurotoxin of claim 57, where the modified neurotoxin exhibits an altered biological

30

activity without exhibiting an altered biological persistence.

62. The modified neurotoxin of claim 57, where the
5 modified neurotoxin exhibits an altered biological activity and an altered biological persistence.

63. The modified neurotoxin of claim 57, where the
10 modified neurotoxin exhibits an increased biological activity and an increased biological persistence.

64. The modified neurotoxin of claim 57, where the
modified neurotoxin exhibits an increased biological activity and a reduced biological persistence.

15

65. The modified neurotoxin of claim 57, where the
modified neurotoxin exhibits a decreased biological activity and a decreased biological persistence.

20 66. The modified neurotoxin of claim 57, where the
modified neurotoxin exhibits an decreased biological activity and an increased biological persistence.

25 67. The modified neurotoxin of claim 57 wherein said
structural modification comprises a leucine-based motif.

68. The modified neurotoxin of claim 57, wherein a unit amount of the modified neurotoxin is more efficient to reduce an exocytosis from a cell than is a unit amount of the naturally existing neurotoxin.

5

Fig. 1



Fig. 2



1	<u>P</u> FV <u>N</u> K <u>Q</u> F <u>N</u> YK	<u>D</u> PVNGVDIAY	<u>I</u> KIPNVG <u>Q</u> M <u>Q</u>	PVKA <u>F</u> KI <u>H</u> NK	IWV <u>I</u> PERDTF
51	TN <u>P</u> EEGD <u>I</u> NP	P <u>P</u> EAK <u>Q</u> V <u>P</u> S	<u>Y</u> YD <u>S</u> T <u>I</u> L <u>S</u> T <u>D</u>	NEKDN <u>Y</u> LK <u>G</u> V	TKL <u>F</u> ER <u>I</u> YST
101	DLGRML <u>T</u> SI	V <u>R</u> G <u>I</u> P <u>F</u> W <u>G</u> S	TIDTELK <u>V</u> ID	TNCIN <u>V</u> I <u>Q</u> PD	GSYRSEEL <u>N</u> L
151	VIIGPSAD <u>I</u> I	<u>Q</u> FECK <u>S</u> FG <u>H</u> E	<u>V</u> L <u>N</u> L <u>T</u> R <u>N</u> G <u>Y</u> G	<u>S</u> T <u>Q</u> Y <u>I</u> R <u>F</u> S <u>P</u> D	F <u>T</u> FG <u>F</u> E <u>E</u> S <u>L</u> E
201	VD <u>T</u> N <u>P</u> LLGAG	K <u>F</u> ATDPA <u>V</u> T <u>L</u>	A <u>H</u> EL <u>I</u> HAG <u>H</u> R	LYGIA <u>I</u> N <u>P</u> NR	V <u>E</u> KVNTNAYY
251	EMSGLEVS <u>F</u> E	ELRTFGGHDA	K <u>F</u> IDS <u>L</u> Q <u>E</u> N <u>E</u>	FR <u>L</u> Y <u>Y</u> Y <u>N</u> K <u>F</u> K	DIA <u>S</u> T <u>L</u> NKAK
301	SIVGT <u>T</u> AS <u>L</u> Q	<u>Y</u> M <u>K</u> N <u>V</u> E <u>K</u> E <u>K</u> Y	L <u>L</u> SED <u>T</u> SG <u>K</u> F	SVDKL <u>K</u> ED <u>K</u> L	<u>Y</u> K <u>M</u> L <u>T</u> E <u>I</u> Y <u>T</u> E
351	D <u>N</u> FV <u>K</u> F <u>F</u> K <u>V</u> L	N <u>R</u> K <u>T</u> Y <u>L</u> N <u>F</u> D <u>K</u>	A <u>V</u> E <u>K</u> I <u>N</u> I <u>V</u> P <u>K</u>	V <u>N</u> Y <u>T</u> I <u>Y</u> D <u>G</u> F <u>N</u>	L <u>R</u> NTN <u>L</u> A <u>N</u> F
401	NG <u>Q</u> NT <u>E</u> I <u>N</u> N <u>M</u>	N <u>F</u> T <u>K</u> L <u>K</u> N <u>F</u> T <u>G</u>	<u>L</u> F <u>E</u> F <u>Y</u> K <u>L</u> L <u>C</u> V	<u>R</u> G <u>I</u> I <u>T</u> S <u>K</u>	

Figure 3

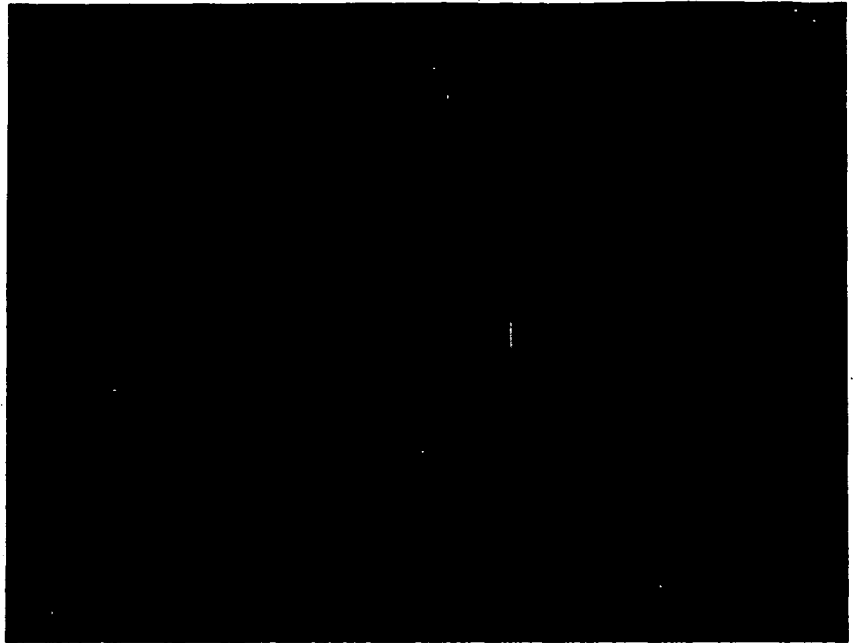


Fig. 4

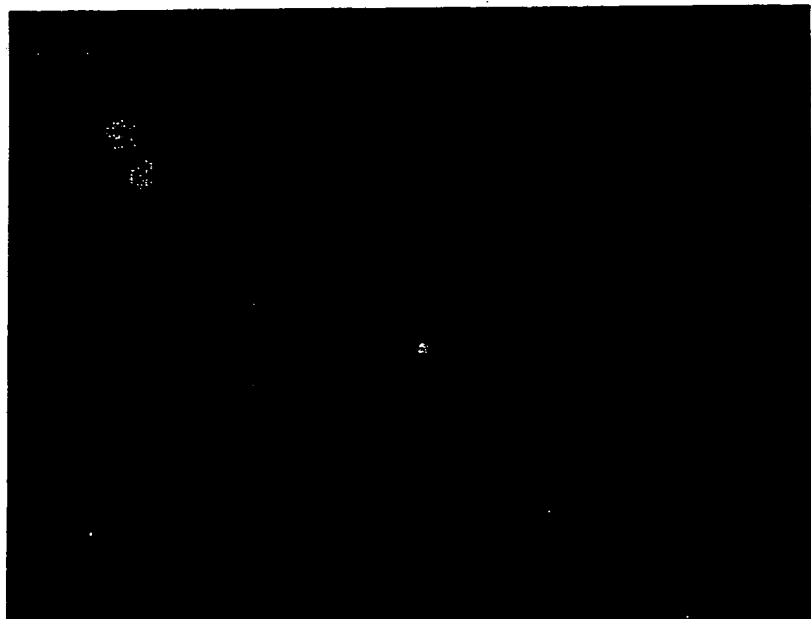
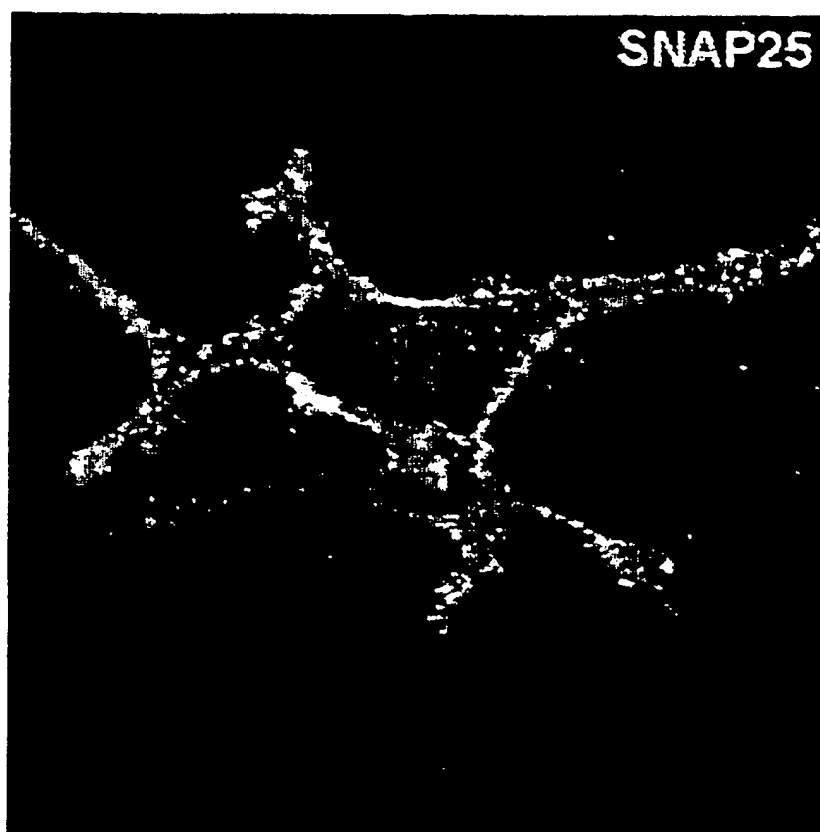


Fig. 7

SUBSTITUTE SHEET (RULE 26)

Fig. 5



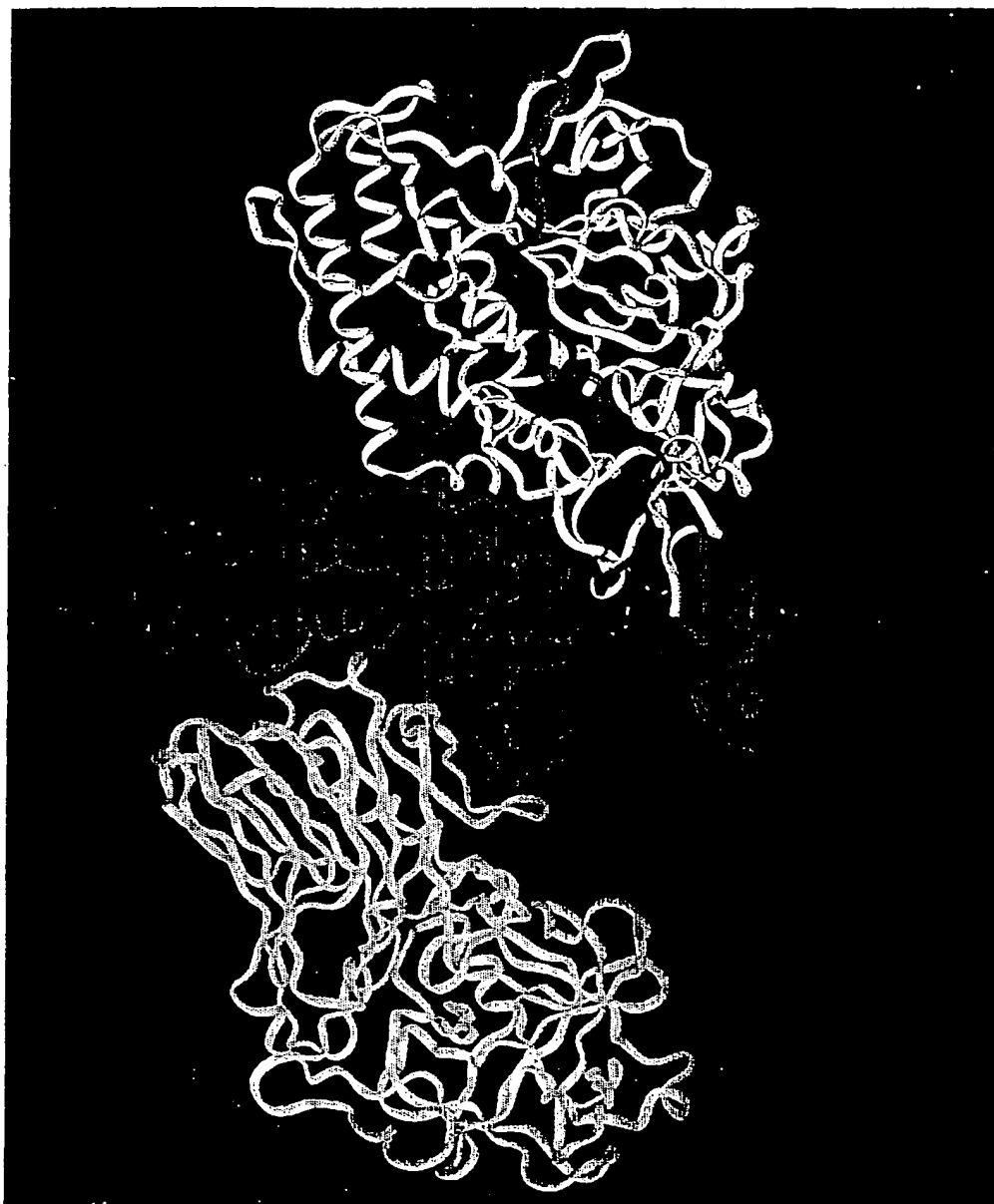


Fig. 6

BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(1)	1	EFVKKQENYKDEVNGVDIAYIKIENVG-QMQPVKAFKIHNKIMVLPEDDTNPEEGDINPPPEAKQVPVSYTD	75
	(1)		MEVTINNENINDEIDNNNIMMEPEFARGTGRYYKAFKIDTETIMVLPEDDTNPEEGDINPPPEAKQVPVSYTD	
	(1)		P NFNY DPI I I P KAFKI KIWIPER TF E YYD	
BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(74)	76	STYLSIDNEKDNITKGVTKLIERIYSTDGRMLITSIVRGIPEMCGSTIDTGLKVIDTNCINVIQPDGSYR-SEE	150
	(76)		PDYLNNDKKNITETOTIKLNRKSKPTGEEKLEMLINIGIPITGDRRVPLPEFNTNIASVTNKLISNPGEVER	
	(76)		YL T K FL M KLF RI S LG LL II GIPF G I E I V E	
BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(148)	151	-----LNLVIGESADIIQFECCKSFCHVNLNTNGYGSQYIRISPDITFGFEESLEVDTNPLGAGKFAIDPA	225
	(151)		KKGIFANLIIISGPGVINENETIDICIONHFASPESEFGIMQKICFEYVSVNNVQENKGASIFNRRCYFSDPA	
	(151)		NLII GP I E G SR GFG IKF PDF F E I F SDPA	
BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(218)	226	VTAAHELIHAGRLYGIAINPNRVKVNNAVYEMSGLEVSEETITFGGHEAKFI DSIQENEFRLYYYNKFRDI	300
	(226)		IIIMHELIHVLIGLYGKVD-DLPVINEKKFEEMQSTDAIOAEELITFGGQPPSIITPSTDKSIYDKVLQNERGI	
	(226)		L L HELIH H LYGI I N FF S I EEL TFGG D I D N FK I	
BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(293)	301	ASTLNKAKSIIG-TTASIQYMKNVKEKYLISDTSKESVCKLKFDKLIHMTETIYTEDTFVKFERVLMNRTLL	375
	(300)		VDRINKVLVCIIDPNININIKNKEKDKYKFVDSSEKYSIDVESFDKLYSMEGETETIAENYKIKTEASTF	
	(301)		LNK I IN KN FKDKY EDS GKFSID FDKLYK L FTE N FKI R SY	
BoNT-A_Halla_LC BoNT-B_Danish_I_LC Consensus	(367)	376	NFDKAVENINIVPKVNTIYDGENLRNTNLAANENSOYTEHNNMNETKLKNFTGLFEFFINLLCVRGIIITSK	447
	(375)		SDSLPPVKIKNLLDNEITITIESEENISDKMEKEVRSOKKAIKQAYEEIIS--KEHLAVINIQMCKSVK---	
	(376)		KI IL YTI DGFNI L F GQN IN F I YKI K I	

FIGURE 8

BirA-SNAP25 Assay of rLC/A Activity

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PCT/US01/23122

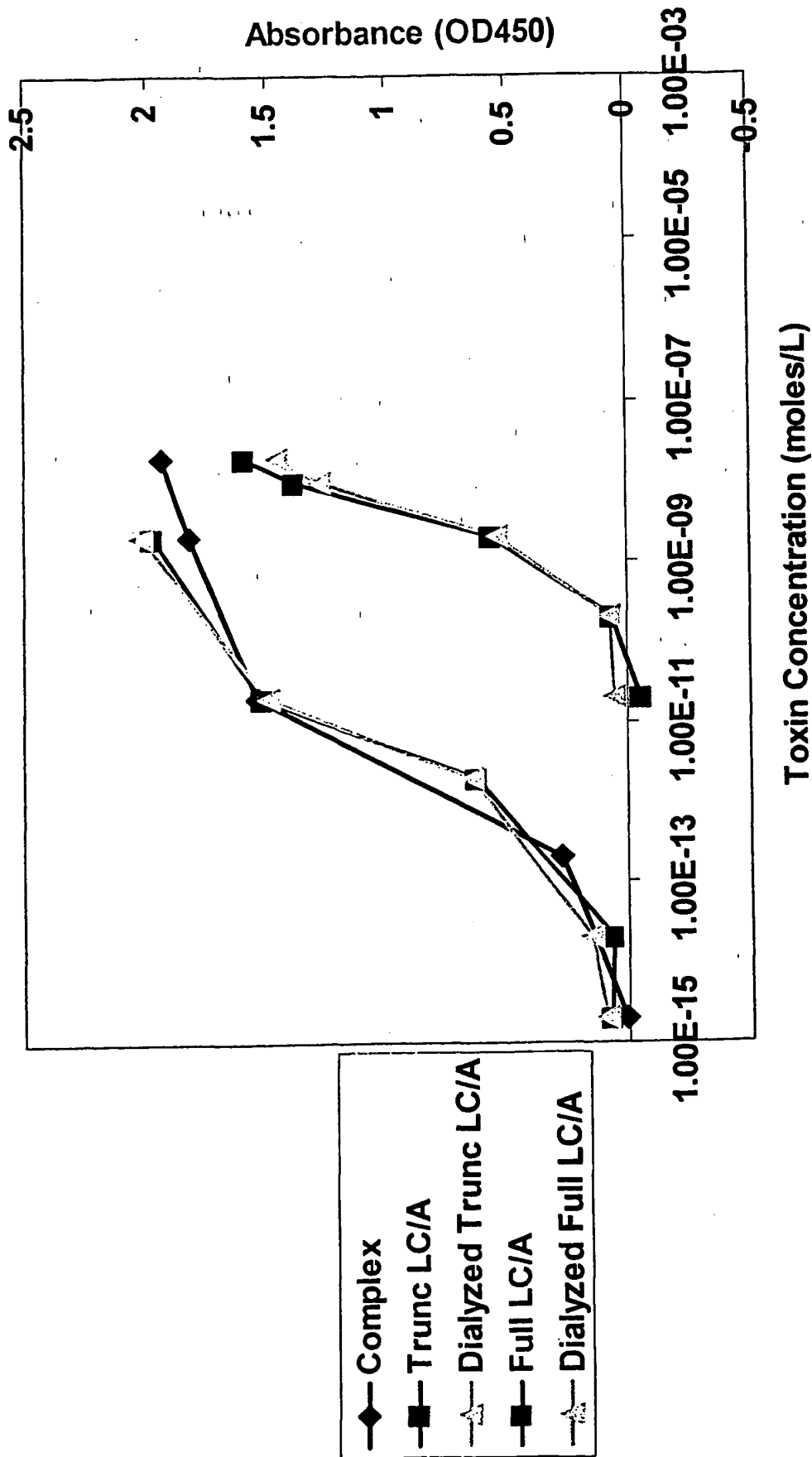


Figure 9

Comparison of LC/A constructs expressed from E. coli for in vitro analysis

	<u>6xHis</u>	<u>S-tag</u>	
Wildtype LC/A			
trun LC/A	MHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAM		PFV NKQFNYKDPV...
N-His LC/A	MHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMG		SFV NKQFNYKDPV...
Wildtype LC/A	...NETKLKNETGL	EEFYKLLCVRGIITSK	
trun LC/A	...NETKLTRAHHHHHH	
N-His LC/A	...NETKLKNETGL	EEFYKLLCVRGIITSK	
		<u>6xHis</u>	

- Truncated LC construct published
 - Kadkhodayan, S. et al. Prot. Exp. Purif. 2000, 19, 125-130
 - Crystal structure reported at IBRCC in Oct. 2000

Figure 10

SEQUENCE LISTING

<110> STEWARD, LANCE E
 FERNANDEZ-SALAS, ESTER
 HERRINGTON, TODD M
 AOKI, KEI R

<120> Leucine-based motif and clostridial neurotoxins

<130> D-2885CIP

<150> US 09/620,840
 <151> 2000-07-21

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 x may be any amino acid or derivatives thereof

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